ASSOCIATIONS BETWEEN MICROTUBULES, PHOSPHOLIPIDS AND INTRACELLULAR MEMBRANES.

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by

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This thesis is dedicated to my parents, Jean and Alan Hargreaves, to whom I am deeply grateful for their constant support and encouragement throughout my academic studies.

"Explore the thought, explain the asking eye"

Alexander Pope

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ABSTRACT

Associations between microtubules, phospholipids and intracellular membranes.

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The aims of the study were to analyse the phospholipids which co-purified with microtubules *in vitro*, to demonstrate that their presence was not an artifact and that they had some function in the regulation of microtubule assembly. All microtubules were prepared by up to five cycles of temperature-dependent polymerization and consisted mainly of tubulin dimer and a number of accessory proteins. Microtubule assembly was monitored by turbidity changes at 37°C after the addition of 0.5mM GTP, or by a colchicine-binding assay.

Chloroform-methanol extracts of microtubules pelleted by centrifugation at one, two and three cycles of purification showed a similar pattern when separated by two-dimensional thin-layer chromatography. The major components were the phosphatides of choline, ethanolamine, serine and inositol along with sphingomyelin and cholesterol. Three-cycle purified microtubules were applied to a phosphocellulose column to separate pure tubulin dimer from accessory proteins. Analysis of total phospholipid phosphate indicated a similar level of phospholipid in both fractions (*ca.* 20 n moles phospholipid per mg protein) which represented, for tubulin, a ratio of 2 moles phospholipid per mole tubulin dimer. Findings from tlc indicated that there were differences in chromatogram patterns of tubulin- and accessory protein-associated phospholipids, which suggested that specific protein-phospholipid associations were involved.

Pre-incubation of one-cycle-purified microtubule extracts (2-3mg/ml) with 0.22 units of phospholipase A (*V.russelli*) or 0.5 units of phospholipase C (*C.welchii*) resulted in 20% to 30% inhibition of the rates and extent of assembly. Phospholipase A effects were reversible in the presence of a lecithin analogue (Dimethyl-DL-2,2-distearoyloxypropyl-2'-hydroxyethylammonium acetate) which competitively inhibits enzyme activity.

Exogenous preparations of phosphatidyl ethanolamine (30μM) enhanced microtubule assembly at a number of protein concentrations (0.4 - 1.8 mg/ml), whereas phosphatidyl choline and lysophospholipids had little or no effect. These observations suggested that changes in the balance of phospholipids could influence microtubule assembly.

An *in vitro* interaction was simulated between microtubules and rat liver smooth endoplasmic reticulum. Polymerization of microtubule proteins (2.4 mg/ml) was inhibited in the presence of various concentrations of added membranes (0.4 - 4.8mg/ml protein). These observations suggested that smooth intracellular membrane material could influence the dynamic equilibrium between polymerized and non-polymerized microtubule proteins.

The observations described above are consistent with the view that specific microtubule protein-phospholipid associations occur which may reflect a number of specific functions of certain phospholipids and intracellular membranes in the regulation of microtubule assembly.

INTRODUCTION

In recent years much interest has been shown in the involvement of microtubules in cellular processes. Work carried out to determine the biochemical properties, structure and function of microtubules has been thoroughly reviewed (Stephens and Edds, 1976; Gaskin and Shelanski, 1976; Dustin, 1978; Roberts and Hyams, 1979; Sakai, 1980; Timasheff and Grisham, 1980; Margolis and Wilson, 1981).

Some specific processes in which microtubules are believed to be involved include the axonal transport of neurotransmitter-containing vesicles and mitochondria (Banks and Till, 1975; Smith, Jarlfors and Cameron, 1975) and the regulation of secretory processes such as insulin secretion (Lacey, Howell, Young and Finck, 1968). Microtubules form an integral part of the cytoskeleton and are, therefore, of importance in the regulation of cell shape (Hsie, O'Neill, Li, Borman, Schroder and Kawashima, 1977) and motility, for example in cilia and flagella (Holwill, 1980; Dentler, 1981). Microtubules are also of fundamental importance in the control of cell division, as they form a major component of the mitotic spindle (Brinkley, Fuller and Highfield, 1975). However, the mechanisms by which microtubules may regulate such processes are still poorly understood.

Microtubule structure consists of regular helical assemblies of their major protein subunits the α- and β-tubulins (Gaskin and Shelanski, 1976). Their cross-sectional diameter, as estimated by electron microscopy, is approximately 25nm (Porter, 1966) with a wall thickness

of 5nm (Bryan, 1974) and a hollow central core of 15nm in diameter. Their length varies considerably *in vitro* up to several microns.

Microtubules are usually composed of thirteen protofilaments, which are postulated to be arranged in the form of a 3-start right-handed helix (Gaskin and Shelanski, 1976).

The tubulin dimer exists in a reversible equilibrium with its α and β subunits (Detrich and Williams, 1978); it has a molecular weight of approximately 110,000 daltons and a sedimentation coefficient of 6S (Shelanski and Taylor, 1967; Johnson and Borisy, 1977; Marcum and Borisy, 1978). The α and β tubulins have similar molecular weights, 55,000 and 53,000 respectively (Bryan and Wilson, 1971; Lee Frigon and Timasheff, 1973), but vary in their amino acid composition, and each can be separated into several minor molecular sub-species by isoelectric focusing (Kobayashi and Mohri, 1977; Forgue and Dahl, 1978; Marotta, Harris and Gilbert, 1977; Saborio, Palmer and Meza, 1978; Nelles and Bamburg, 1978). Tubulin is an acidic protein and the isoelectric points of its molecular sub-species vary over a narrow pH range between 5.2 and 5.4. Tubulin may be a glycoprotein as it has been reported that a carbohydrate moiety present in tubulin preparations *in vitro* is inseparable from tubulin on a basis of molecular weight or net charge (Feit and Shelanski, 1975). There is also chemical evidence which shows a level of approximately 9 moles total sugar per mole tubulin dimer (Prus and Mattisson, 1979).

Tubulin can be purified *in vitro*, from a variety of both neural and non-neural tissues, by a number of methods which include ammonium sulphate precipitation (Weisenberg, Borisy and Taylor, 1968; Weisenberg and Timasheff, 1970), vinblastine precipitation (Marantz, Ventilla and Shelanski, 1969) and temperature-dependent recylcization (Shelanski, Gaskin and Cantor, 1973; Borisy, Marcum and Allen, 1974; Larsson, Wallin and Edström, 1976). The method used in the present study was exclusively that of temperature-dependant recyclization. Such procedures depend on the ability of tubulin and associated proteins to self-assemble into microtubules at 37°C in the presence of magnesium ions and GTP over the pH range 6.4 - 6.9, and to disassemble at 4°C into tubulin dimers.

One of the most popular procedures of temperature-dependent recyclization incorporates glycerol into the assembly medium (Shelanski *et al.*, 1973). The presence of glycerol stabilizes tubulin and increases the yield of microtubules (Shelanski *et al.*, 1973; Cleveland, Hwo and Kirschner, 1977). However, tubulin prepared in this way may contain up to five moles of tightly bound glycerol (Detrich, Berkowitz, Kim and Williams, 1976) which may be responsible for the observed differences in the number of free sulphydryls present and the appearance of tubulin aggregates at 4°C (Kirschner, Williams, Weingarten and Gerhardt, 1974; Borisy, Marcum, Olmstead, Murphy and Johnson, 1975). Therefore, glycerol-stabilized microtubules may be 'unnatural' unless glycerol is considered to be substituting for the influence of a similar

effect *in vivo* which may be lost by microtubule preparation in the absence of glycerol.

Microtubules prepared by temperature-dependent recyclization are sensitive to the action of a large variety of compounds. Microtubule assembly in both crude and purified preparations is particularly sensitive to low concentrations of free calcium (Olmstead and Borisy, 1973; Olmstead and Borisy, 1975) colchicine and the Vinca alkaloids (Weisenberg and Timasheff, 1970; Lambier and Engelborghs, 1978; Brewer, Loike and Horwitz, 1979; Loike, Brewer, Sternlicht, Gensler and Horwitz, 1978; Wallin and Larsson, 1979; Castle and Crawford, 1978). Other microtubule inhibitors include methyl benzimidozolyl carbamates (Hoebeke, Van Nijen and De Brabander, 1976), griseofulvin (Wehland, Herzog and Weber, 1977), maytansine (Remillard, Rebhun, Howie and Kupchan, 1975), barbiturates (Ventilla and Brown, 1976; Edström, Hansson, Larsson and Wallin, 1975), antibiotics (Akiyama, Tanaka, Tanaka and Nonomura, 1978), psychotropic drugs (Poffenbarger and Fuller, 1977), rotenone (Marshall and Himes, 1978) trialkyltin compounds (Tan and Kumar Das, 1978) and many others.

Relatively few compounds have been discovered which promote microtubule assembly *in vitro* under normal buffer conditions. Such compounds are glutamate (Suzaki, Sakai, Endo, Kimura and Shigenaka, 1978), spermine (Lee, Tweedy and Timasheff, 1978), ascorbic acid (Boxer, Vanderbilt, Bonsib, Jersild, Hang and Baehner, 1979) and Taxol (Schiff, Fant and Horwitz, 1979). Taxol is particularly interesting because, like colchicine, it inhibits cell

division (Schiff and Horwitz, 1980; Schiff et al., 1979). However, it has a stabilizing effect on microtubules and reduces sensitivity to cold and Ca^{2+}. This observation, together with the recent discovery of a population of cold-stable microtubules in brain extracts (Webb and Wilson, 1980), suggests that taxol could be mimicking an endogenous compound which determines the equilibrium between cold-stable and cold-labile microtubules which may have different functions within the cell.

Microtubules prepared by temperature-dependent recylization have, in addition to tubulin, a number of associated 'accessory' proteins and enzyme activities. The nature and amount of accessory proteins seems largely dependent on the differences between extraction procedures of different workers and whether glycerol is present or not. Another important factor could be the levels of proteolytic enzymes present during tissue fractionation and the length of time elapsing between slaughter of the animal and microtubule extraction.

There are two major categories of accessory proteins, known as 'tau' (Weingarten, Lockwood, Hwo and Kirschner, 1975; Witman, Cleveland, Weingarten and Kirschner, 1975; Cleveland et al., 1977) and the high molecular weight microtubule-associated proteins (MAPs)(Murphy and Borisy, 1975; Murphy, Vallee and Borisy, 1977; Sloboda, Dentler and Rosenbaum, 1976).

Tau factor has been characterised by gel electrophoreseis as four closely-related polypeptides with molecular weights in the range 55,000 - 62,000 and is

required in stoichiometric amounts to promote microtubule assembly *in vitro*. It has a sedimentation coefficient of 2.6S and is phosphorylated by a microtubule-associated protein kinase (Witman *et al.*, 1975; Cleveland *et al.*, 1977).

MAPs have been characterised as two polypeptides, MAPs 1 and 2, of molecular weights in the range 250,000 to 350,000 daltons which are required in stoichiometric amounts to promote microtubule assembly *in vitro* (Murphy and Borisy, 1975; Sloboda *et al.*, 1976; Murphy *et al.*, 1977). Herzog and Weber (1978) showed that purified preparations of MAPs and tau were both able to stimulate microtubule assembly with comparable activity *in vitro*. However, in the case of MAP-stimulated microtubules these authors observed the presence of characteristic side-arm structures which were absent from the tau-stimulated microtubules.

The importance of MAPs and tau *in vivo* is not yet certain, but they are known to form an integral component of the mitotic spindle in cultured fibroblasts (Sherline and Schiavone, 1977), and of microtubule networks in a variety of cells and tissues (Bulinski and Borisy, 1980a, 1980b). In addition, MAPs are known to bind *in vitro* to various DNA sequences and show, in the case of MAP 2, a high specificity for pericentromeric DNA (Corces, Salas, Salas and Avila, 1978; Wiche, Corces and Avila, 1978; Corces, Manso, De La Torre, Avila, Nasr and Wiche, 1980; Villasante, Corces, Manso-Martinez and Avila, 1981). These findings suggest that MAP 2 is a likely candidate

to mediate the attachment of the kinetochore to the mitotic spindle during cell division.

Microtubule assembly *in vitro* occurs by a nucleated condensation mechanism which consists of distinct nucleation and elongation stages (Johnson and Borisy, 1977; Gaskin and Shelanski, 1976). The nucleation step involves the formation of intermediate structures such as 30S rings, discs and spirals from 6S tubulin and accessory proteins (Kirschner *et al.*, 1974; Kirschner, Honig and Williams, 1975; Burns, 1978) which act as nucleation centres for subsequent microtubule formation. It is believed that accessory proteins play an important role in nucleation by binding several tubulin dimers per accessory protein molecule, to effectively increase the local concentration of tubulin (Weingarten *et al.*, 1975). It is also evident that accessory proteins, although not an absolute requirement for elongation, stabilize microtubules against depolymerization and, therefore, shift the equilibrium to support microtubule formation (Cleveland *et al.*, 1977).

Microtubule elongation proceeds by the addition of 6S tubulin molecules (Johnson and Borisy, 1977) or ring structures (Zeeberg, Cheek and Caplow, 1980; Pantaloni, Carlier, Simon and Batelier, 1981) to the existing ends of microtubules until a 'steady state' is reached where the net rates of tubulin assembly and disassembly are equal (Margolis and, Wilson, 1978). Under those conditions there is a net flux or treadmill of tubulin dimers along the microtubule from a proximal primary assembly site to the distal disassembly site at the

opposite end of the microtubule (Margolis and Wilson, 1978, 1981). Although treadmilling of tubulin has not yet been demonstrated *in vivo*, it could be utilized in a number of ways to control the intracellular flow of a variety of macromolecules.

Under normal buffer conditions, *in vitro*, microtubule assembly will not occur in the absence of added nucleotides although polymerization can be induced by abnormally high concentrations of magnesium ions and glycerol (Shelanski *et al.*, 1973). GTP is hydrolysed during assembly and is important in the treadmilling of assembled tubulin in microtubules at steady-state *in vitro* (Margolis and Wilson, 1978). There is now considerable evidence to suggest that GTP hydrolysis is not a requirement for microtubule assembly, as assembly occurs in the presence of non-hydrolysable analogues of GTP (Sandoval and Weber, 1980; Margolis, 1981; Cote and Borisy, 1981; Carlier and Pantaloni, 1981), but is an absolute requirement for the treadmilling of tubulin at steady-state *in vitro*.

GTP is found in association with at least two distinct binding sites known as the exchangeable or E-site and the non-exchangeable or N-site (Penningworth and Kirschner, 1977; Zeeberg and Caplow, 1978). It is believed that only the E-site GTP is hydrolysed during microtubule assembly and that the binding of GTP is dependent on free sulphydryl groups (Mann, Giesel and Fasold, 1978). GTP may be acting as an allosteric effector at the E-site during microtubule assembly (Penningworth and Kirschner, 1977). More recent evidence has suggested that there may

be two mutually-exclusive E-sites (Hamel and Lin, 1981).

A GTPase activity is detectable at the ends of microtubules during assembly, which is attributed to the tubulin molecule itself. This activity is enhanced in the presence of MAPs, but can also be induced by abnormal buffer conditions (David-Pfeuty, Erickson and Pantaloni, 1977; David-Pfeuty, Laporte and Pantaloni, 1978).

A variety of other enzyme activities are also present in microtubule preparations. ATPases have been detected in some preparations (Gaskin, Kramer, Cantor, Adelstein and Shelanski, 1974; Gelfand, Gyoeva, Rosenblat and Shanina, 1978; Banks, 1976) which led to the proposal of an analogous function for microtubule-associated ATPase to that of dynein in cilia and flagella (Mohri, 1976). However, a contradictory report describes this activity as a contaminant from membrane-bound microsomal ATPases (Banks, 1976) and whether or not these ATPases are an intrinsic component of microtubules remains unclear.

Cyclic AMP-dependent protein kinases are also associated with microtubules (Goodman, Rasmussen, Dibella and Guthraw, 1970; Lagnado and Tan, 1975; Sloboda, Rudolph, Rosenbaum and Greengard, 1975; Sheterline and Schoefield, 1975; Sheterline, 1977; Sheterline, 1978a; Sheterline, 1978b; Ikeda and Steiner, 1979). Some kinase activities have been detected which are intrinsic to tubulin and others appear to be present in accessory proteins.

The calcium-dependent regulator protein, known as calmodulin (Marcum, Dedman, Brinkley and Means, 1978;

Nishida, Kumagai, Ohtsuki and Sakai, 1979; Kumagai and Nishida, 1979; Job, Fischer and Margolis, 1981; Schliwa, Euteneur, Bulinski and Izant, 1981; Burke and DeLorenzo, 1981) induces microtubule disassembly *in vitro* (Job *et al.*, 1981) and at the same time stimulates endogenous phosphorylation. These observations, together with the fact that phosphorylation of MAPs reduces microtubule assembly (Jameson and Caplow, 1981), suggest that Ca^{2+}/calmodulin-mediated phosphorylation of microtubule proteins could play a major part in the regulation of microtubule-mediated phenomena.

Other microtubule-associated proteins which are believed to play a regulatory role in microtubule assembly are acid and alkaline phosphatases (Larsson, Wallin and Edström, 1979), adenylate cyclase (Mohri, 1976), and a post-translational tubulin-tyrosine ligase (Raybin and Flavin, 1977; Rodriguez and Borisy, 1978; Kumar and Flavin, 1981).

Tubulin-tyrosine ligase activity reaches a maximum at 14 - 16 days post-fertilization in the chick embryo, which correlates well with the increase in availability of hydrolysable tyrosine and the availability of the α-tubulin carboxyl terminus for *in vitro* tyrosination (Rodriguez and Borisy, 1978). Changes in the extent of C-terminus modication may be of significance to the participation of microtubules in various developmental events.

Of particular interest to the present study are the existence of a diglyceride kinase (Daleo, Piras and Piras, 1974, 1976) and phosphodiesterases (Quinn, 1975; Van de Berg,

1975) in association with microtubule proteins.

A phospholipase C-type phosphodiesterase specific for phosphatidyl inositol is detectable in the soluble protein fraction from brain and thyroid (Quinn, 1975). It exists in two distinct forms when complexed with different combinations of tubulin. A high molecular weight form comprises a colchicine-labile interaction between two tubulin dimers and one enzyme molecule, whereas the low molecular weight form involves a complex between one enzyme and one β-tubulin molecule. Although the enzyme was shown not to be a structural component of microtubules, phospodiesterase activity has been localized cytochemically to microtubules in sensory nerves of an insect (Van de Berg, 1975). It is likely that further purification of microtubule proteins would lose this component although it is probably important in the regulation of microtubule assembly or function *in vivo*. Many other functionally-important enzymes could be 'loosely' associated with microtubules in a similar manner and lost on purification.

Daleo *et al*., (1974, 1976) reported the consistent presence of a diglyceride kinase activity and a number of phospholipids (Daleo *et al*., 1974) in association with microtubules prepared from different sources. It is unlikely that the activity observed was a contaminant from the soluble protein high-speed supernatant, from which it was derived by vinblastine precipitation, due to its differences in sensitivity to pH, ions, detergent, ouabain and sedimentation behaviour (Daleo *et al*., 1976). The

diglyceride kinase activity could not be separated from a protein kinase by sedimentation and several of their properties such as nucleotide specificity, heat inactivation and sensitivity to trypsin digestion, were similar. Although these findings suggest that one enzyme may catalyse phosphorylation of both diglyceride and protein, this possibility is ruled out by the lack of inhibition of the phosphate transfer to one substrate when the other substrate is present at a saturating concentration. The kinase activities, which were shown not to be associated with tubulin dimer or oligomer by sedimentation on a sucrose gradient, are probably specifically associated with microtubules as suggested by the distinct activities recovered in microtubules, compared to the multiplicity of activities recovered in the corresponding high speed supernatant. The function of the diglyceride kinase is still not known, but presumably it is concerned in some way with the metabolism of phospholipids which may have some function in microtubule formation.

Lagnado and Kirazov (1975), on investigating the incorporation of ^{32}P into rat and chick brain <u>in vivo</u>, discovered that almost half of the total bound radioactivity recovered was in the form of phospholipid phosphate. This was associated mainly with phosphatidyl ethanolamine (PE), phosphatidic acid (PA) and protein-bound phosphoserine phosphate. The high metabolic activity of microtubule-bound ^{32}P which they observed may reflect a function of phosphorylation of phospholipid molecules in the regulation of microtubule assembly. In 1977 Kirazov

and Lagnado observed that myo-inositol interacted with brain microtubules *in vitro* by reducing the polymerizability of tubulin and stabilizing microtubules against Ca^{2+} and low temperature. They proposed a physiological role for inositol in the microtubule-mediated metabolism of membrane phosphoinositides. However, Pickard and Hawthorne (1978) showed that the inositol effect on microtubules could be mimicked by a variety of other sugars and cyclitols, which suggested that the inositol effect could be non-specific.

Other workers have reported the presence of a number of phospholipids associated with microtubules from bovine splenic nerve (Banks, 1976) rat and chick brain (Daleo *et al*., 1974; 1977). The major phospholipids were identified as PE and phosphatidyl choline (PC), although all the authors claimed that other phospholipds were present. Nagle and Bryan (1975), Bryan (1975) and Daleo *et al*., (1977) showed that incubation of commercial preparations of phospholipases with tubulin extracts inhibited microtubule assembly in the case of phospholipase A_2 (Nagle and Bryan, 1975; Daleo *et al*., 1977) and promoted assembly in the case of phospholipase D (Daleo *et al*., 1977). There was disagreement as to whether phospholipase C promoted (Daleo *et al*., 1977; or inhibited (Bryan, 1975) microtubule assembly. Such differences could have arisen due to differences in the quality of enzyme preparations used, buffer conditions or the microtubule protein composition which was not clearly described in either publication. The phospholipase

effects were not due to a contaminating protease or GTPase in the commercial enzyme preparations (Daleo et al., 1977). In addition, microtubule assembly was apparently enhanced on the removal of microtubule-associated phospholipids by detergent treatment. This effect could be partially reversed by the addition of an extract of commercial phospholipids or re-addition of microtubule-associated phospholipids (Daleo et al., 1977). However, it was not clear in that work to what extent the detergents used interfered with the viscometric and turbidimetric measurements of microtubule assembly. Work by Bryan (1975) indicated that the inhibition of microtubule assembly by colchicine involved an interaction between colchicine and a lipid-protein binding site on the tubulin dimer.

All of these results suggest that phospholipids in microtubule preparations may have a functional role in determining the association of tubulin molecules during microtubule assembly, and the final dimensions of the assembled microtubules. This regulation could be brought about by structural association between phospholipids with tubulin or accessory proteins or both. The rate of turnover of the microtubule-associated phospholipds may have some influence on the regulation of a variety of microtubule-associated enzymes.

An alternative explanation for these observed associations could be a functional interaction between microtubules and membranes. The occurrence of structural links within the cell between microtubules and intracellular

membranes, particularly mitochondria and neurotransmitter-containing vesicles, has been demonstrated (Smith, 1971; Smith, Jarlfors and Cayer, 1977; Smith et al., 1975). These links are in the form of structural cross-bridges with components of both the microtubule and the membrane in question (Smith et al., 1977). Connections between microtubules and mitochondria in axons of amnacoete larvae of the lamprey (Petromyzon marinus) have been shown, by computed statistical analysis and comparison with simulated axons, to occur at a significantly higher level than if they were purely random (Smith et al., 1975).

Other observations of microtubule-membrane associations include an association between microtubules and the golgi complex in embryonic chick spinal ganglion cells (Hinek, 1977); between microtubules and mitochondria and endoplasmic reticulum in guinea pigs (Makita and Kiwaki, 1978); between microtubules, microfilament networks and pore complexes in the endoplasmic reticulum of cultured tumour cells (Chemnitz and Salmberg, 1978); between microtubules and membranes in cilia and flagella (Dentler, 1981).

In vitro studies also suggest that microtubule-membrane interactions can occur. For example Caron and Berlin (1979) showed that microtubule proteins were taken up into phospholipid vesicles which resulted in the formation of multilamellar structures "indicative of a strong lipid-protein interaction". These had a striking resemblance to the organisation of some intracellular membranes but, as microtubule formation did not occur

during these experiments, further work is required to prove unequivocally that microtubules are able to orientate membranes and thereby regulate membrane-mediated processes.

Other evidence in support of microtubule-membrane interactions includes the isolation of a membrane-bound tubulin which differs slightly in chemical properties from the soluble cytoplasmic form (Bhattacharrya and Wolff, 1976; Nath and Flavin, 1978). Tubulin has also been identified as a major component of synaptic vesicle membranes (Zisapel, Levi and Gozes, 1980), ciliary membranes (Stephens, 1977) and as a smaller component of a variety of other membranes. In addition, colchicine affects the fluidity of membrane structures, possibly by binding to membrane and/or microtubule protein (Altsteil and Landsberger, 1977). Interactions between microtubules and membranes have also been observed using flourescein-labelled membranes and rhodamine-labelled tubulin (Becker, Oliver and Berlin, 1975) as well as interactions between membranes and anti-tubulin antibodies (Walters and Matus, 1975).

The presence of tubulin in a variety of membranes led Feit and Shay (1980) to investigate the possibility of an alternative form of tubulin assembly into membranes rather than microtubules. These authors found that tubulin in high-speed supernatants of bovine brain were able to polymerize into membrane-like structures at 37°C in the presence of 10^{-4}M colchicine. The assembly reaction was inhibited by low temperature,

although not reversible at $4^{o}C$, and 1mM $CaCl_2$ or $10^{-4}M$ maytansine. The membranous material contained 80% tubulin and 8% MAPs and, as is the case with assembled microtubules, bound a much smaller proportion of colchicine in its assembled form compared to the disassembled material. Although further work is required to improve the *in vitro* conditions for tubulin assembly into membranes, this system could prove very useful to study the function of membrane-bound tubulin in the regulation of interactions between membranes and the cytoskeleton.

Taken together all of these findings point strongly towards the possibility of tubulin-lipid interactions which may be of great importance in the regulation of microtubule-mediated and membrane-mediated processes. It may be that microtubules are able to interact with membranes and exert some control over membrane function or alternatively, membranes may regulate microtubule assembly and microtubule-associated phenomena.

In summary, it was known at the start of the present study that a number of phospholipids and phospholipid-metabolizing enzymes were present in microtubule preparations. It was also known that exogenous phospholipases affected microtubule assembly *in vitro* and that microtubules were often associated with membrane structures *in vivo*. The aims of the present study were to confirm the presence of phospholipids in pig brain microtubules, to attain a more comprehensive identification than that achieved by previous workers,

to demonstrate and quantify their association with highly-purified microtubule proteins and to ascertain whether specific protein-phospholipid associations occurred. Having achieved these objectives, it was then necessary to show whether microtubule-associated phospholipids were involved in the regulation of microtubule assembly and also, whether their presence might reflect an *in vivo* interaction between microtubules and intracellular membranes.

MATERIALS

All of the materials and reagents used were obtained as Analar grade from British Drug Houses (BDH), Poole, Dorset, with the exception of the following:-

(a) In thin-layer chromatography and phospholipid extractions, chromatography grade chloroform, methanol, acetone and glacial acetic acid were used. These reagents, together with the following:

acrylamide, specially purified for electrophoresis

amino napthol sulphonic acid (ANSA), clinical reagent

caesium chloride, specially purified for ultra-centrifuge work

dextran (molecular weight 170,000), grade A

1,4-di(phenyloxazolyl) benzene (POPOP), 'scintran'

Folin-Ciocalteau reagent

glutaraldehyde, 25% aqueous solution

iodine, resublimed

lysophosphatidyl choline (LPC), egg grade I

lysophosphatidyl ethanolamine (LPE), egg grade I

p-nitrophenyl phosphate, crystalline sodium salt

osmium tetroxide (osmic acid), solid

phosphatidyl choline (PC), egg grade I

phosphatidyl ethanolamine (PE), egg grade I

phosphatidyl inositol (PI), sodium salt from wheat germ

silica gel 60 (Merck), 20 x 20cm glass plates pre-coated to a layer thickness of 0.25mm (without fluorescent indicator)

toluene, scintillation grade

Triton X-100, scintillation grade

All from BDH.

(b) benzidine, 95% pure

bovine serum albumin (BSA)

4-bromophenacyl bromide, crystalline

cardiolipin (DPG), bovine heart

colchicine, crystalline

cytochrome c, type III

ethylene diamine tetra acetic acid (EDTA), disodium salt

ethylene glycol-bis(β-amino-ethyl ether) N,N' -tetra acetic acid (EGTA), crystalline

gamma globulins, Cohn fraction II

guanosine 5'triphosphate (GTP), grade II-s

2-(N-morpholino)ethane sulphonic acid (MES), crystalline

ninhydrin, crystalline

phosphatidic acid (PA), lyophilised sodium salt from egg yolk

phosphatidyl serine (PS), ox brain

piperazine-N,N'-bis(2-ethane sulphonic acid)(PIPES), crystalline free acid

sodium dodecyl sulphate (SDS), 95% pure

tris(hydroxymethyl)methylamine (TRIS), trizma base

All from Sigma Chemical Company, Poole, Dorset.

(c) adenosine 5'-monophosphate (AMP)

glucose-6-phosphate

sphingomyelin (SP), highly-purified from ox brain

thiamine pyrophosphate

All from Koch-Light, Colnbrook, Bucks.

(d) copper grids for electron microscopy, 300-mesh Spurr resin kit

Both from Agar Aids, Stansted, Essex.

(e) DEAE cellulose filters, DE81 (2.5cm diameter) phosphocellulose, P11

From Whatman, Maidstone, Kent.

(f) (ring C-methoxyl-^{3}H) colchicine

From Amersham Radiochemicals Limited, Amersham, Bucks.

(g) 7-^{14}C-tyramine hydrochloride was a gift from Dr. A.J.George, School of Pharmacy, Liverpool Polytechnic.

(h) collodion was a gift from Dr. P. Sheterline, Department of Histology and Cell Biology, Liverpool University.

METHODS

1. GENERAL POINTS ON METHODOLOGY

1.1 Centrifugation procedures

(a) All centrifuge speeds are calculated for the centre of the tubes.

(b) Unless otherwise stated in METHODS, centrifugation was performed in an MSE 8 x 50ml rotor (34^{o} angle), when a volume of 20ml or over was involved, or an MSE 10 x 10ml rotor (20^{o} angle) for smaller volumes.

1.2 Cleanliness of glassware

(a) All glassware was washed with Pyroneg detergent and rinsed at least three times with fresh changes of distilled and deionised water.

(b) When test tubes were to be used for inorganic phosphate assays they were given an extra 2 or 3 rinses with distilled deionised water to ensure removal of traces of phosphate.

(c) Glassware used in phospholipid extraction and analysis was, in addition to the above treatment, rinsed inside with methanol and then chloroform before use.

1.3 Spectrophotometry

(a) Unless otherwise specified in METHODS absorbance readings were made from samples placed in glass cells with 1cm light path.

(b) Absorbance values were read against a distilled water blank and the appropriate reagent blank mean values (from triplicate readings) was subtracted from each sample reading.

Enzyme and protein assays.

(a) All assays were carried out at least in triplicate.

(b) Specific activities were calculated as micromoles of substrate hydrolysed per milligram protein per hour.

1.4 Statistics

(a) Quantitative results were expressed as mean ± SEM or mean ± standard deviation (SD), whichever was more appropriate, as explained in RESULTS.

(b) When necessary (as indicated in RESULTS) significance of results were estimated by student's "t" test. The level of probability is indicated in the legend of the appropriate figures.

2. PREPARATION OF MICROTUBULES

Reagents:-

(a) Reassembly buffer (RB) - 100mM piperazine-N,N'-bis (2-ethane sulphonic acid) (PIPES)
1mM ethylene glycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA)
0.5mM $MgSO_4$
pH 6.8 adjusted by addition of NaOH.

(b) Guanosine 5'-triphosphate (GTP)

Method:-

The procedure used involved slight modifications to the method of Larsson, Wallin and Edström (1976). Pig brains were obtained from the local abattoir within two hours of slaughter. Superficial blood vessels and meninges were removed immediately and then the whole brain tissue was cut with scissors into small pieces in RB on ice. All subsequent operations, unless otherwise stated, were carried out on ice. The brain tissue pieces were then rinsed twice with RB (approximately 50ml per brain). Then approximately 1ml RB per wet weight brain tissue was added to the washed brain tissue which was then homogenised in a glass tube by ten passes of a tissue disperser at 25,000 r.p.m. followed by four passes with a motor-driven glass-teflon homogeniser set at 600 r.p.m.

The resultant crude homogenate was then centrifuged at 75,000g for one hour at 2°C to pellet whole cells, cell debris and membranes. The pellet was discarded and the supernatant was incubated with 0.5mM GTP at 37°C for 20 - 25 minutes to initiate microtubule formation. Microtubules were then pelleted by centrifugation at 45,000g for 45 minutes at 30 - 37°C.

The microtubule pellets were then rinsed twice with RB and resuspended until homogeneous by vigorous glass-glass homogenisation in a volume of RB equivalent to $^1/_5$ of the previous supernatant volume. The resuspended pellet was incubated on ice for 30 minutes to depolymerise the microtubules. The resultant cloudy suspension was

centrifuged at 20,000g for 20 - 30 minutes at 2°C to remove cold-stable aggregates. The clear supernatant was then incubated with GTP and centrifuged as described above. This completed a further cycle of microtubule purification.

Microtubules were prepared in this manner through up to five cycles of purification. Purified microtubule pellets were stored at -70°C or under liquid nitrogen (-175°C) for up to six weeks.

3. MEASUREMENT OF IN VITRO MICROTUBULE ASSEMBLY.

3.1 Turbidity measurement

Reagents:-

50mM GTP

Method:-

Microtubule assembly was monitored by change in turbidity measured at 350nm as described by Gaskin, Cantor and Shelanski (1974). Measurements were made in a Pye Unicam SP1800 or a Perkin Elmer 551 spectrophotometer. Each instrument was fitted with a temperature-regulated cell housing and linear chart recorder, but the latter had an automatic sample-changer and cell-programmer with a capability of monitoring five samples simultaneously.

In a typical experiment microtubule pellets were resuspended in RB to the desired protein concentration (normally 2 - 3 $mg.ml^{-1}$) by glass-glass homogenisation, incubated on ice for 30 minutes and then clarified by centrifugation at 20,000g for 20 - 30 minutes at 2°C as described in PREPARATION OF MICROTUBULES. One ml of the

clear supernatant was usually prewarmed at 37°C for 2 minutes and then added to a glass cuvette (0.5cm light path), containing 10µl of 50mM GTP (thus giving a final concentration of 0.5mM GTP), in the spectrophotometer cell housing at 37°C. Changes in turbidity were then recorded on a linear chart recorder until microtubule polymerization was complete. Any modifications to this scheme are described in RESULTS.

3.2 Colchicine-binding assay

Reagents:-

(a) Sodium phosphate-sucrose buffer - 83.75mM $NaH_2PO_4.2H_2O$
0.5mM $MgSO_4$
1.25M sucrose
pH 6.8 adjusted with NaOH

(b) Filtration buffer - 67mM $NaH_2PO_4.2H_2O$
100mM KCl
pH 6.8 adjusted with NaOH

(c) ^{3}H-colchicine - 0.36mM, specific activity 2.8Ci/m.mol

(d) Stock diluted ^{3}H-colchicine - 5mM unlabelled colchicine containing 0.1ml of ^{3}H-colchicine per ml.
Final concentration, 4.04mM
Final specific activity, 24.75 mCi/m.mol.

(e) Whatman (DE81) DEAE cellulose filter discs (2.5cm diameter)

(f) Triton scintillation fluid - triton X-100 (1 part)
toluene (2 parts)
0.5% w/v PPO (2,5-diphenyl oxazole 'Scintran')
0.099% w/v POPOP (1,4-di-phenyloxazolyl benzene 'Scintran').

Method:-

The procedure adopted was based on the filter disc assay described by Borisy (1972) and modified by Tsé and Doherty (1980) with further modifications. Colchicine-binding assays were carried out on soluble supernatant proteins. These were obtained by centrifugation of polymerized microtubules and membranes at 35,000g for 45 minutes at 37°C from microtubule-membrane interaction experiments described in RESULTS Section 4.

The supernatants, containing microtubule protein in RB, were carefully decanted and then diluted by a factor of 1:4 with sodium phosphate-sucrose buffer. Thus the final assay concentrations were 67mM sodium phosphate, 1M sucrose, 0.5mM $MgSO_4$, 20mM PIPES and 0.2mM EGTA.

Aliquots of 0.5ml of diluted supernatant microtubule protein were incubated in triplicate with 10µl of stock diluted ^3H-colchicine for four hours at 37°C. The incubation was terminated by placing the samples in an ice bath.

Filtration was performed using a stack of four filter discs sealed inside a millipore filter housing. The filter housing was clamped in position and connected

to an overhead reservoir made from a 20ml syringe housing. Filter stacks were prepared from four filter discs which were moistened with distilled deionised water, compressed and then dried overnight in an oven at 60°C. The filter stacks were remoistened with filtration buffer immediately before assembly of the filter apparatus.

After colchicine-binding, each 0.5ml sample was diluted into 10ml of filtration buffer in the reservoir. The reservoir contents were immediately filtered at a rate of approximately 3 seconds per ml by suction from a water pump. Each filter stack was then washed four times with 10ml of filtration buffer. The rate of filtration decreased on subsequent washes. Care was taken to prevent the filter stacks from running dry between consecutive washes. After the final wash, each filter stack was allowed to run dry for about 10 seconds, after which it was transferred to a plastic scintillation vial containing 10ml of Triton scintillation fluid.

All radioactivity was eluted from the filter stacks by incubation at room temperature for 24 hours. Radioactivity was then determined in a Kontron (Intertechnique) liquid scintillation counter. Background counts were determined from filter stacks washed with unlabelled buffer and non-protein-bound radioactivity was measured from filter stacks which had been washed with filtration buffer containing labelled colchicine. The latter did not exceed background activity by more than 30 c.p.m.

4. FURTHER PURIFICATION OF MICROTUBULE PROTEINS BY PHOSPHOCELLULOSE CHROMATOGRAPHY.

Reagents:-

(a) 0.5N NaOH

(b) 0.5N HCl

(c) Phosphocellulose (Whatman P11)

(d) Reassembly buffer (RB) - as described in Section 1.1

(e) Column buffer - 20mM PIPES
1mM EGTA
0.5mM $MgSO_4$
pH 6.8, adjusted with NaOH

Method:-

The method was based on the procedure described by Weingarten et al., (1975) and included some of the modifications of Williams and Detrich (1979).

4.1 Preparation of phosphocellulose column

10 - 20g of phosphocellulose resin was washed in 500ml of 0.5N NaOH for no more than 5 minutes. The phosphocellulose was allowed to settle and then the NaOH was carefully decanted away. The slurry was then mixed with 500ml of distilled water, allowed to settle again, and the water decanted away. This procedure was repeated until the supernatant pH reached a value below 10. The water was then replaced by 500ml of 0.5N HCl and the phosphocellulose allowed to settle for five minutes. The HCl was decanted away and the phosphocellulose washed repeatedly with water as described above, until the pH of the supernatant reached a value above 3.

The supernatant was decanted and the wet-settled phosphocellulose (50 - 100ml) was then washed twice with two volumes of RB. After the second supernatant had been decanted away, the slurry was titrated to pH 6.8 with NaOH. This was followed by four washes with two volumes of column buffer each time and a titration to pH 6.8 between each wash, until the pH remained at 6.8 (usually between the third and fourth wash).

Most of the supernatant except for about 20ml, was poured away and the slurry poured into a LKB water-jacketed chromatography column. The column was sealed airtight and approximately one litre of column buffer was run through it, under the force of gravity, to allow the bed to settle. The column was then sealed off at both ends by clamping screws until required for protein separation. Before a chromatographic separation was carried out a further 500ml of column buffer was run through the column and the pH of the eluent was checked to be exactly the same as that of the buffer in the reservoir above the column. During this period the column temperature was equilibrated at 0 - 4^{o}C by means of a liquid-circulator and an ice bath. This temperature was maintained throughout the subsequent chromatographic separation of microtubule proteins.

4.2 Chromatographic separation of tubulin from accessory proteins.

Three-cycle-purified microtubule pellets, prepared as described in METHODS Section 2, were resuspended by vigorous

glass-glass homogenisation in column buffer to a concentration around 5 - 10 $mg.ml^{-1}$. The suspension was incubated on ice for 30 minutes and then clarified by centrifugation at 25,000g for 20 - 30 minutes at $2^{o}C$. Enough of the suspension was retained for phospholipid analysis (described in Section 7), electrophoresis (described in Section 5) and protein assay (described in Section 11), while the rest was applied to the equilibrated column via a three-way valve. The amount of protein loaded onto the columns was kept within the limits of 0.4 - 1.0mg per ml bed volume. Chromatography was carried out at 0 - $4^{o}C$ as described above and the flow rate, which varied with bed volume, was 15 - 30 $ml.hour^{-1}$. Fractions of approximately 2ml volume were collected in plastic scintillation vial inserts on a LKB Ultrorac fraction collector. Protein content of each fraction was estimated by the absorbance at 280nm measured with a Pye Unicam SP30 spectrophotometer. After absorbance measurement the collected fractions were stored on ice until the chromatographic procedure was completed.

Tubulin was eluted in the void volume, usually between fractions 10 and 30, while the accessory proteins were retained in the column. Elution of accessory proteins was achieved by the addition of 1N NaCl (in column buffer) to the column, via the three-way valve, as soon as the last fraction of the tubulin peak had been collected (see RESULTS).

All the void volume fractions (containing tubulin) and the NaCl-eluted fractions (containing accessory proteins)

were pooled separately and, along with unchromatographed microtubule protein from the same original preparation, were then dialysed in visking tubing for 12 - 14 hours at 4°C against two changes of RB (500ml each time). After dialysis the pooled fractions were transferred to glass tubes and the visking tubing washed through with 4ml of cold RB to remove residual material.

Small aliquots of dialysed material were taken for protein assays and electrophoresis to determine recovery and efficiency of the chromatographic separation of microtubule proteins (see RESULTS). The remaining bulk of the dialysed material was either used directly for phospholipid extraction (as described in Section 6.2) or stored frozen at -70°C until required. Lipid extracts were further analysed by thin-layer chromatography (tlc) or phospholipid phosphate assay (Section 7) as shown in RESULTS.

5. ELECTROPHORESIS OF MICROTUBULE PROTEINS

Reagents:-

(a) Sample buffer - 10% w/v glycerol
5% w/v 2-mercaptoethanol
2% w/v sodium dodecyl sulphate (SDS)
62.5mM tris(hydroxymethyl) methylamine (TRIS)
pH 6.8 adjusted with HCl

(b) Bromophenol blue - 0.25% w/v in sample buffer

(c) 2-mercaptoethanol

(d) 30% glycerol-acrylamide - 29.2% w/v acrylamide
0.8% w/v N,N'-methylene bisacrylamide
75% w/v glycerol

(e) 30% water-acrylamide - 29.2% w/v acrylamide
0.8% w/v N,N'-methylene bisacrylamide

(f) 4% acrylamide in lower gel buffer - a 1:6.5 dilution of 30% water acrylamide:lower gel buffer

(g) 4% acrylamide in upper gel buffer - a 1:6.5 dilution of 30% water acrylamide:upper gel buffer

(h) 15% acrylamide in lower gel buffer - a 1:1 dilution of glycerol acrylamide:lower gel buffer

(i) Upper gel buffer - 0.4% w/v SDS
0.5M TRIS
pH 6.8 adjusted with HCl

(j) Lower gel buffer - 0.4% w/v SDS
1.5M TRIS
pH 8.8 adjusted with HCl

(k) N,N,N',N'-tetramethyl ethylene diamine (TEMED)

(l) 10% w/v ammonium persulphate

(m) Running buffer - 0.1% w/v SDS
0.192M glycine
25mM TRIS

(n) Fixing solution - 15.4% w/v trichloroacetic acid

3.4% w/v sulphosalicylic acid

30% v/v methanol

(o) Staining solution - 0.25% w/v coomassie brilliant blue R250

7.2% v/v glacial acetic acid

45.4% v/v methanol

(p) Destaining solution - 30% v/v ethanol

10% v/v glacial acetic acid

(q) Preserving solution - 30% v/v ethanol

10% v/v glacial acetic acid

10% v/v glycerol

Methods:-

Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis (SDS-PAGE) procedures were based on the method of Weber, Wehland and Herzog (1976) with some modifications.

5.1 Preparation of gel

Electrophoresis was always performed on horizontal polyacrylamide slab gels which usually consisted of a 4 - 15% gradient gel and a 4% stacking gel. To prepare the gradient gel 20ml of both 4% water-acrylamide and 15% glycerol-acrylamide in lower gel buffer were mixed with 40µl of 10% ammonium persulphate and degassed for one minute. Then, immediately prior to pouring the gradient, 15µl of TEMED were added to each solution to catalyse polymerization of the gel.

The gradient was poured with the aid of a MSE gradient former and mixing of the gradient was achieved with a motor-driven glass stirrer. The activated acrylamide solutions were poured into the wells of the gradient former, the heavier solution nearest to the outlet. The stirrer was switched on and at the same time the gradient former outlet was opened to allow the solutions to pour into the gel mould. The connecting valve between the two wells of the gradient former was immediately opened to allow mixing of the two acrylamide solutions. Thus, as the pouring procedure continued, the 4% acrylamide became a progressively larger constituent of the pouring solution. In order to obtain an undisturbed linear gradient the outlet tubing tip was held in a position just above the surface of the gradient as it rose within the gel mould.

The mould assembly consisted of two glass plates (12 x 26cm) separated by a 1.5mm thick rubber gasket. During gel preparation the mould assembly was maintained in a vertical position and sealed around its edges with bulldog clips. One of the glass plates was completely smooth, whereas the other one had oblong-shaped blocks extruding about 1mm into the mould space in the region of the stacking gel. Thus, when the mould was dismantled after complete polymerization of the gel the final gel, resting on the smooth glass plate, had a stacking gel with a line of twelve slots, of approximately 20µl capacity, into which the protein samples were loaded.

The gradient gel was left to polymerize (about one hour at room temperature) after which the surface liquid

was removed with a syringe. Care was taken not to damage the surface of the gradient gel at this stage. The surface of the polymerized gradient gel was then rinsed with 5ml of a mixture of lower gel buffer (one part) and distilled water (three parts) containing 0.25% w/v ammonium persulphate and 0.25% w/v TEMED. The wash solution was then removed carefully with a syringe. This made the gradient gel surface more adhesive towards the stacking gel which was subsequently poured into the mould.

The stacking gel consisted of 20ml of 4% water-acrylamide in upper gel buffer, which was activated with 80µl of 10% ammonium persulphate and 30µl of TEMED as described above. The activated solution was then carefully poured through a funnel until the remaining mould space was completely filled. At this point the mould was tilted, with the inlet uppermost, to remove any air bubbles trapped along the length of the mould. If necessary the mould was then topped up again before the gasket inlet was sealed down. The completed gel was then left to complete polymerization in a refrigerator for 8 - 16 hours. The mould was then carefully dis assembled by prising away the slotted glass plate with a spatula and pulling away the rubber gasket. The gel was now ready for use for electrophoretic separation of microtubule proteins.

5.2 Treatment of protein samples before electrophoresis

Microtubule protein in solution in RB either at various stages of microtubule purification or from various experiments, as indicated in RESULTS, were freeze-dried. The freeze-dried protein was redissolved in electrophoresis sample buffer to a concentration of 2 - 5mg/ml. Samples were boiled for two minutes and then rapidly chilled on ice. Then 40µl of 2-mercaptoethanol and 40µl of bromophenol blue were added per ml sample buffer and mixed thoroughly. A sample containing a mixture of low (11,000 - 70,000) and high molecular weight (50,000 - 260,000) protein standards were treated in a similar manner and electrophoresed in parallel.

5.3 Electrophoresis

Electrophoretic separation of microtubule proteins was performed on a LKB Multiphor apparatus. Continuity between the gel and running buffer was achieved by means of filter wicks, which were set up according to the manufacturers' instructions. The gel, prepared as described in Section 4.1, was pre-electrophoresed at 30mA for 20 minutes to remove any traces of impurities. Proteins treated in sample buffer, as described in Section 4.2, were loaded at concentrations within the range 2 - 100µg per sample slot.

The current was then applied initially at 20mA until the bromophenol blue marker dye bands had moved to the interface between the stacking gel and the gradient gel. The current was then stepped up gradually to 100mA or

sufficient to maintain a field strength of 5 - 10 volts cm^{-1} across the gel. Electrophoresis was continued until the marker dye front had migrated to within 0.5cm of the end of the gel. The distance moved by the dye front from the centre of each well, and the exact length of the gel perpendicular to the centre of each well were measured before the gel was removed from the apparatus.

The gel was then placed in fixing solution for one hour followed by two hours in staining solution at room temperature. It was then processed through several changes of destaining solution to remove background stain from the gel. Finally, the gel was soaked for one hour at room temperature in preserving solution before it was wrapped in cellophane and dried on a glass plate. When the gel was completely dry (several hours at room temperature) the distance moved by each polypeptide band in the electrophoresis pattern was measured from the centre of the well to the centre of the appropriate band. The exact length of the gel, perpendicular to the middle of each well, was also measured. Relative mobilities of the polypeptide bands were calculated from the equation:-

$$\text{Relative mobility (Rf)} = \frac{\text{distance moved by protein} \times \text{original length of gel}}{\text{distance moved by dye front} \times \text{gel length after drying}}$$

A standard curve was plotted, between R_f and $\log_{10}$ molecular weight of the protein standards, from which the apparent molecular weight of microtubule proteins could be estimated.

Where quantification was required stained gels, or photographs of gels, were scanned with a Zeiss Mk.I chromatogram spectrophotometer at 700nm or 415nm respectively. The tubulin peaks and the accessory protein profiles were cut out from the constant-weight recorder chart paper and weighed to quantify the composition of microtubule protein extracts. Such determinations were carried out within a total protein concentration range of 2 - 10µg per gel, over which tubulin's densitometric peak area was observed to increase linearly with protein concentration.

6. EXTRACTION OF MICROTUBULE-ASSOCIATED PHOSPHOLIPIDS

Phospholipids were extracted, from microtubule pellets or suspensions of microtubule protein in RB, in chloroform-methanol according to the procedures of Folch, Lees and Sloane-Stanley (1957) or Bligh and Dyer (1959) with some modifications. The Bligh and Dyer procedure was used only on microtubule proteins fractionated by phosphocellulose chromatography where indicated in RESULTS Section 2. Otherwise the procedure of Folch *et al.*, (1957) was always used.

6.1 Folch et al., procedure

Reagents:-

(a) Chloroform-methanol (2:1, by volume)

(b) Pure solvent upper phase- chloroform:methanol: 0.017% w/v aqueous $MgSO_4$ (3:48:47, by volume)

(c) 0.017% w/v aqueous $MgSO_4$

Method:-

The procedure used involved a scaling-down of the method of Folch et al., (1957). Microtubule pellets at various stages of microtubule purification or aliquots of microtubule protein from phosphocellulose chromatography (Section 4) were freeze-dried prior to phospholipid extraction.

Freeze-dried protein, which was still associated with RB components, was subjected to continuous and vigorous glass-glass homogenisation on ice for four minutes in a volume of 2ml of chloroform-methanol. The homogenate was immediately filtered at $4^{o}C$ through a fat-free filter paper, previously washed with chloroform-methanol, into a glass-stoppered vial. The homogeniser was then washed with a further 1 ml of chloroform-methanol which was then passed through the same filter as above. The filter was finally washed through with another 1 ml of chloroform-methanol. In most cases (see RESULTS) this extraction procedure was repeated a second time on the residue remaining in the filter which was allowed to dry first and then scraped off with a clean scalpel.

Then, 0.8ml of 0.017% $MgSO_4$, was added to the filtrate (4ml) in the glass-stoppered vial and mixed by inversion. The vial contents were then either centrifuged, in a MSE bench centrifuge, at 2,400 r.p.m. for 20 minutes or left to stand on ice for about one hour. During this period the aqueous (upper) and organic phases separated without interfacial fluff.

The upper phase was then almost completely removed by aspiration leaving only a small amount in contact with the lower phase. The surface of the lower phase was then washed with 1ml of pure solvent upper phase which was gently layered onto it by pipette. Pure solvent upper phase was removed by aspiration and this procedure repeated a total of three times to remove all non-lipid contaminants which separate into the upper phase (Folch et al., 1957). The final wash was completely removed at the expense of a small amount of the lower phase.

The organic phase, which contained phospholipids, was then evaporated to dryness in a vacuum chamber at 0 - 4°C. The dried lipid extract was stored in a dessicator at -20°C until required for tlc (Section 7.1) or phospholipid phosphate assay (Section 7.3), whereupon it was resuspended in chloroform-methanol by vigorous vortex mixing.

6.2 Bligh and Dyer procedure

Reagents:-

(a) Chloroform-methanol (1:2, by volume)

(b) Chloroform

(c) Distilled, deionised water

Method:-

The procedure used was based on that described by Bligh and Dyer (1959) and was only employed in the extraction of phospholipids from post-dialysis microtubule proteins from phosphocellulose chromatography where indicated in RESULTS Section 2.

The method involved homogenisation of protein in solution in RB, with chloroform-methanol to form a monophasic system where the ratio of chloroform:methanol: water was 1:2:0.8 by volume. This homogenate was then diluted with water and chloroform to produce a biphasic system in which the solvent ratios were 2:2:1.8. Lipids are retained in the lower chloroform layer and non-lipids are extracted in the methanol-water upper phase (Bligh and Dyer, 1959).

Three volumes of chloroform-methanol were added to 0.8 volumes of microtubule protein in RB and subjected to vigorous homogenisation in a MSE blender, set at maximum speed, for two minutes on ice. One volume of chloroform was then added and the mixture blended for another 30 seconds. One volume of water was added and the mixture again blended for a further 30 seconds. A minimum total volume of around 20ml was required in the homogenisation vessel used in these extractions.

The homogenate was then filtered, at 0 - 4^{o}C to reduce evaporation, through a fat-free filter paper into a glass tube. A second extraction was then performed on the filter residue and filter paper which were homogenised in the same volumes of chloroform-methanol etc., as for the first extraction. The homogenisation vessel was then washed through with 5ml of chloroform-methanol, which was filtered, and the second filter finally washed through with an additional 5ml of chloroform-methanol. The filtrates from both extractions were pooled and left to settle into two phases at 0 - 4^{o}C.

The upper phase was then removed completely by aspiration at the expense of a small amount of lower phase solvent. The chloroform phase was evaporated to dryness either at 0 - 4°C in a vacuum chamber or at 40°C under a stream of nitrogen gas. Dried extracts were stored in a dessicator at -20°C and resuspended in an appropriate volume of chloroform-methanol (2:1, by volume) when required for tlc or phospholipid phosphate assay as described in Sections 7.1 and 7.3 respectively.

7. SEPARATION AND ANALYSIS OF MICROTUBULE-ASSOCIATED PHOSPHOLIPIDS

7.1 Two-dimensional thin-layer chromatography

Reagents:-

(a) Solvent system I - chloroform:methanol:7M aqueous ammonia (230:90:15, by volume)

(b) Solvent system II - chloroform:methanol: glacial acetic acid:water (250:74:19:3, by volume)

(c) Chloroform-methanol (2:1, by volume)

(d) Silica gel 60 - commercially pre-coated thin-layer chromoplates, 20 x 20 x 0.025cm

(e) 20% w/v aqueous ammonium sulphate

(f) Iodine crystals

(g) Lipid standards - Phosphatidyl choline (PC), lysophosphatidyl choline (LPC) phosphatidyl ethanolamine (PE), lysophosphatidyl ethanolamine (LPE), phosphatidyl serine (PS), phosphatidyl inositol (PI), sphingomyelin (SP), phosphatidic acid (PA), cardiolipin (DPG), cholesterol (CH).

Method:-

Reproducibility of calculated relative mobilities (R_f) was enhanced by the employment of standard chromatography conditions and, wherever possible, chromatograms were run in parallel to reduce differences due to variations in temperature, humidity, etc. All plates were activated in a dry oven at 110°C for one hour and then stored in a large dessicator until used on the same day. Dried lipid residues, prepared as described in Section 6.1 and 6.2, were resuspended in chloroform-methanol (2:1, by volume) by vigorous vortex mixing. In a typical separation 10 - 50µl of the chloroform-methanol extract which contained 10 - 250 nanomoles of microtubule-associated phospholipid phosphate (assayed as described in Section 7.3) were applied with a microcap applicator to form an origin spot 2cm in from each edge of the bottom left-hand corner of the activated plate. The origin spot, the diameter of which did not exceed 5mm, was dried in a stream of cool air from a fan-heater.

The applied extract was then separated by ascending chromatography in a sealed glass tank, which measured approximately 25 x 25 x 5cm and was pre-equilibrated with 120ml of solvent system I, with two plates to each tank. Each chromatogram was run for a distance of approximately 15cm after which the solvent front was lightly marked in pencil and the plate was dried in a stream of cool air for about 40 minutes to remove traces of solvent. The same plate was then positioned at right-angles to the original direction of chromatography, with the origin spot at the bottom, and separation carried out in the same manner as described above, in fresh glass tanks containing solvent system II. The plate was finally dried in a stream of hot air until all traces of solvent were removed. A further run in the second dimension was sometimes employed as this improved the resolution of separation and reduced 'tailing' on the chromatogram spots.

Each chromatogram was placed in a sealed glass tank with a few crystals of iodine for 15 - 20 minutes. During this period the unsaturated lipids developed as yellow spots. Iodine was used as a non-specific locating agent due to its ability to attach at any unsaturated carbon bond. The chromatogram spots were traced onto acetate sheets and then allowed to fade before the plate was subjected to charring, where a permanent record was required, or specific spray reagents (as described in Section 7.2) for identification purposes.

Chromatograms were charred when a photograph was required to be taken or for densitometry (see RESULTS).

After chromatography and iodine treatment, as described above, the plate was sprayed with 20% w/v ammonium sulphate until uniformly translucent and then dried in a stream of hot air. It was then charred by incubation in a pre-heated oven for one hour at 210°C. Diacyl phospholipids and cholesterol developed as black spots with a limit of sensitivity within the range 0.5 - 2.0µg per chromatogram. However, PA, DPG and the monoacyl phospholipids (LPC and LPE) were not as sensitive to this treatment. This was presumably due to either a lower number of carbon atoms per molecule (compared to the diacyl phospholipids) or to excessive spreading or tailing of spots during chromatography. These lipids did, however, react with iodine in most cases and LPC was sometimes detectable under ultraviolet light by fluorescence even after charring had been carried out. Charred chromatograms were scanned in a Zeiss mark I chromatogram spectrophotometer at 415nm where indicated in RESULTS. Chromatograms were scanned on the reverse side of the plates as this gave a more uniform background. Where quantification was required, densitometric peak areas were cut out and weighed.

During experiments involving identification by R_f (see RESULTS) chromatograms were run in quadruplicate and in parallel with chromatograms containing lipid standards. R_f's were calculated as the distance moved from the origin to the centre of the spot along its axis of migration divided by the distance moved by the solvent front for each dimension. Preliminary identification was thus obtained by comparison of R_f values of unknown microtubule-

associated lipids with those of the lipid standards. These findings were then confirmed by the use of specific spray reagents as described in Section 7.2 A 'fingerprint' of the lipid standards was obtained by running chromatograms of the standard mixtures in which a different standard was excluded from each mixture.

Having established the identification of microtubule-associated phospholipids it was desirable to economise on tlc plates and reagents by running four chromatograms on each plate wherever convenient (see RESULTS Section 2). This was achieved by dividing each plate into four equal squares (10 x 10cm), which were lightly marked in pencil, and applying samples into each corner of the plate (1.5cm in from each edge).

Two chromatograms were run simultaneously on the same plate in the first dimension before the plate was dried in a stream of cool air. The plate was then inverted and the other two chromatograms run in the same dimension. After the plate was dried again in a stream of cool air it was placed in a fresh glass tank, which contained solvent system II, at an angle of 90^{o} to its original orientation. Then all four chromatograms were run in the second dimension in the same manner as described above. The resultant chromatogram pattern was identical to that achieved when a whole plate was used and the chromatogram components were sharper. However, due to the shorter distances over which these chromatograms were run (about 7 - 8cm) the spots moved closer together and were more difficult to analyse by densitometry. Only DPG showed any

difference in R_f when separated in this sytem. Its R_f in the first dimension was identical to its separation over a longer distance, but in the second dimension the DPG spot moved much closer to the solvent front than was previously observed.

7.2 Specific spray reagents

Chromatograms, developed as described in Section 7.1 and recorded onto acetate sheets, were treated with the following spray reagents for further identification of specific chromatogram components.

(i) Dragendorff reagent.

Reagents:-

(a) Stock solution - 2.6g bismuth carbonate and 7g sodium iodide dissolved by boiling for a few minutes in 25ml glacial acetic acid. Then after 12 hours the precipitated sodium acetate was filtered off. 20ml of the red-brown filtrate was then mixed with 80ml ethylacetate and 0.5ml water and stored in the dark.

(b) Spray solution - 10ml of stock solution was mixed with 100ml of glacial acetic acid and 240ml of ethyl acetate.

Method:-

The chromatogram was sprayed lightly with 5 - 10ml of spray solution (Tyihák, 1964).

A yellow colour appeared within a few minutes on one spot which was identified as PC. When a series of dilutions of standards spotted onto a tlc plate were tested with the spray, it was observed that the reaction was most sensitive for PC (5 - 10µg) although there was colour development with SP and PE standards at higher concentrations.

(ii) Ninhydrin reagent

Reagents:-

0.5% w/v ninhydrin in acetone

Method:-

Ninhydrin spray reagent was used to detect amino-containing compounds separated by chromatography (Dittmer and Lester, 1964). Chromatograms were sprayed lightly and then incubated in a pre-heated oven at 90° C for 15 minutes. A pink colouration developed in amino-containing lipids.

The reaction was positive for PS, PE and LPE at concentrations below 10µg per chromatogram. The other standards, excluding cholesterol, reacted with the spray only at much higher concentrations.

(iii)Benzidine reagent

Reagents:-

(a) Reagent I - 5ml household bleach

50ml benzene

(b) Reagent II - 10% w/v benzidine
One crystal of potassium iodide
50% v/v ethanol

Method:-

The method was that described by Bischel and Austin (1963). The chromatogram was lightly sprayed with reagent I and then air-dried in a fume cupboard to remove traces of chlorine. Then the same chromatogram was sprayed with reagent II and left at room temperature in a fume cupboard for a few minutes. Sphingolipids developed as blue spots with a sensitivity of less than 10µg. This spray was also observed to react with PE and PC at similar concentrations.

(iv) Cholesterol-locating reagent.

Reagents:-

Concentrated H_2SO_4:glacial acetic acid (1:1, by volume).

Method:-

The chromatogram was sprayed uniformly with the above reagent and the incubated in a pre-heated oven at 90°C for about 15 minutes. Cholesterol developed as a deep purple spot and there was no apparent cross-reaction with any of the phospholipid standards (from J.N.Hawthorne, personal communication).

(v) Anthrone reagent

Reagents:-

(a) 10% v/v H_2SO_4

(b) 1% w/v anthrone in benzene

Method:-

The chromatogram was sprayed uniformly with 10% H_2SO_4 followed by 1% anthrone in benzene and then incubated in a pre-heated oven at 100°C for 10 minutes. A blue colour develops in spots containing cerebrosides (Van Gent, Roseleur and Van der Bijl, 1973) and there is no interference from other lipids.

(vi) Bial's reagent.

Reagents:-

Spray reagent - 10ml of 10% w/v diphenylamine in ethanol mixed with 37ml of concentrated HCl and 80ml of glacial acetic acid

Method:-

The chromatogram was sprayed lightly with the above spray reagent and then incubated at 100°C for 5 - 10 minutes whilst covered with a clean glass plate of the same size (20 x 20cm). Glycolipids develop as blue spots (Jatzkewitz, 1960).

(vii)Molybdate reagent.

Reagents:-

Solution 1 - 40.11g of MoO_3 in one litre of 25N H_2SO_4; dissolved by gentle boiling.

Solution 2 - 1.78g of powdered molybdenum in 500ml of solution 1, boiled gently for 15 minutes and then cooled. The solution

was then decanted from any undissolved residue.

Method:-

Equal volumes of solutions 1 and 2 were mixed and then the combined solution was mixed with two volumes of water. The final solution was greenish yellow in colour. If too little water was used it was blue; if too much, yellow. Chromatograms were sprayed lightly with this solution until uniformly damp. Compounds containing phosphate ester showed up immediately as blue spots on a light blue-grey background. The intensity of the colour increased on standing, until eventually (after a few hours) the background darkened to obscure the spots. A wide range of compounds including PC, PE, SP, PS, PI, PA and DPG standards all gave a positive reaction. Cholesterol did not react. The method used was based on that described by Dittmer and Lester (1964).

7.3 Assay of total phospholipid phosphate

Reagents:-

(a) 70% v/v aqueous perchloric acid

(b) 5% w/v aqueous ammonium molybdate

(c) Aminonaptholsulphonic acid reagent (ANSA)

(d) Stock KH_2PO_4 ($5mg.ml^{-1}$)

Method:-

The procedure used involved a scaling-down of the method of Bartlett (1959). No more than 30µl of microtubule-associated phospholipids (prepared as described in Section 3), resuspended in chloroform-methanol, were added to

300µl of 70% perchloric acid in each assay tube. Control samples included (i) a phospholipid extraction from RB containing 0.5mM GTP (ii) the addition of pure chloroform-methanol to perchloric acid and (iii) the addition of phospholipid extract after the subsequent acid incubation described below.

All samples were incubated for three hours in a preheated oven at 180°C, in parallel with a series of standards containing 0 - 5µg of KH_2PO_4. The samples were allowed to cool and then 2ml of distilled deionised water was added followed by 0.1ml of 5% w/v ammonium molybdate and 0.1ml of ANSA. Each sample was thoroughly vortex-mixed and then incubated at 90°C for about 30 minutes to allow colour development. The samples were cooled and their absorbance read at 830nm in a Pye Unicam SP30 spectrophotometer.

8. ELECTRON MICROSCOPY OF MICROTUBULE PROTEINS AND MEMBRANES

8.1 Coating of electron microscope specimen grids

Reagents:-

(a) Copper grids (300-mesh, 3.05mm diameter)

(b) 1.7% collodion in amyl acetate

Method:-

Very fine forceps were used to place the grids, with their dull side facing upwards, in the bottom of a flat-bottomed Buchner funnel (8cm diameter) filled with distilled water. The funnel was filled to the brim and the water surface was cleaned by rolling a glass rod, washed with acetone, across it.

One drop of collodion solution was applied by pasteur pipette to the centre of the water surface and left for 20 - 30 seconds during which time a collodion film was formed. This film was picked up by rolling it around a clean glass rod, taking with it any remaining dust from the water surface. Another drop of collodion was then applied and left 20 - 30 seconds until the film had formed. The water was then gradually drained away from the funnel, through the outlet tube, leaving the collodion film in contact with the grids.

The coated grids were left to dry at room temperature for about 40 minutes and then picked up carefully with fine forceps and transferred to a Petri dish, lined with filter paper, for storage.

8.2 Direct negative staining

Reagent:-

2% w/v aqueous uranyl acetate

Method:-

Aliquots (approximately 5µl) of polymerized or non-polymerized microtubule proteins with or without added membranes or phospholipids, as described in RESULTS, were applied to the film on separate coated grids which were held with fine forceps. After an interval of 15 - 20 seconds the applied specimen was stained with 2 - 3 drops of uranyl acetate, which were applied from a syringe fitted with a millipore filter, for five seconds. The stained specimen was then washed with a few drops of distilled water and blotted dry on a filter paper.

Stained specimens were stored in separate Petri dishes lined with filter paper until they were examined in a Phillips EM802 transmission electron microscope.

8.3 Preparation of specimens for thin sectioning

Reagents:-

(a) 2% v/v glutaraldehyde in RB

(b) 1% w/v aqueous osmium tetroxide

(c) 0.5% w/v aqueous uranyl actate

(d) 70% v/v ethanol

(e) 96% v/v ethanol

(f) 100% ethanol

(g) Spurr's resin - The following reagents were added together, in a fume cupboard, in the order shown below. Each reagent was thoroughly mixed before the next addition. 1g ERL 4206 (vinyl cyclohexene dioxide), 6g DER 726 (diglycidyl ether of polypropylene glycol) 26g NSA (nonenyl succinic anhydride) 0.4g S1 (dimethyl amino ethane, DMAE).

Method:-

Microtubules and membranes, either separately or together as explained in RESULTS, were pelleted by centrifugation at 35,000g for 45 minutes at 37°C. The

pellets were rinsed twice with RB at 37°C and then fixed for 2 - 4 hours at 37°C in 2% glutaraldehyde solution. They were then rinsed with three changes of RB before they were post-fixed for one hour at 4°C in 1% aqueous osmium tetroxide. The fixed pellets were rinsed with three changes of distilled water and incubated overnight at 4°C with 0.5% aqueous uranyl acetate after which they were washed twice for twenty minutes in distilled water.

The pellets were then dehydrated by a series of two ten-minute washes in 70% ethanol, followed by two washes in 95% ethanol and finally, three washes in 100% ethanol. Instead of decanting the third ethanol wash it was mixed with an equal volume of Spurr's resin and left for 10 minutes. More resin was then added to a level of one volume of ethanol to two volumes of resin. Finally, the ethanol-resin mixture was decanted and replaced with pure resin.

The fixed pellets were then incubated at 4°C overnight in the pure resin which was replaced by fresh resin on the following day. The pellets were sliced into pieces as explained in RESULTS. The sliced pieces were then placed into octagonal slots in a rubber mould and the slots topped up with pure resin. The mould was then heated at 60°C for about ten hours to harden the resin. The specimens were then ready for thin sectioning.

8.4 Thin sectioning

Resin blocks which contained the fixed specimens were removed from the rubber mould and trimmed down with

a razor blade to the level of the specimen. A trapezoid shape of length 0.1 - 0.5mm was then cut, from an area of resin containing the specimen, to extend out from the rest of the trimmed resin block surface. Silver-gold sections were cut with the aid of a LKB ultramicrotome and a glass knife.

Sections were floated on distilled water, contained in a water bath constructed from surgical tape around the knife edge. The same sections were then stretched, by wafting them with a cotton wool bud soaked in chloroform, before they were picked up by adhesion to the collodion film of a coated copper grid.

8.5 Staining of thin sections

Reagents:-

(a) Lead citrate - Prepared by mixing 1.33g lead nitrate and 1.76g sodium citrate in 30ml of distilled water for one minute and then every 3 - 4 minutes for the next 30 minutes. 8ml of 1N NaOH were then added and the solution made up to 50ml with distilled water and used no less than two days later. It was important to store this solution in the dark and free from agitation.

(b) 2% w/v aqueous uranyl acetate - stored refrigerated in the dark in a syringe fitted with a millipore filter to remove large crystals when dispensed.

Method:-

The staining procedure was carried out on a wax plate enclosed within a Petri dish. Pellets of NaOH were placed on the wax plate; each was moistened with distilled water and left for five minutes. Drops of lead citrate were then applied to the wax plate (no more than four drops per Petri dish) and the coated copper grids placed on the stain, specimen side down, for no more than five minutes. The specimens, held with fine forceps, were rinsed for five seconds with 0.02N NaOH and then 30 seconds with distilled water before being blotted dry on a filter paper.

Several drops of uranyl acetate were applied to another wax plate in a covered Petri dish and the same grids placed specimen side down and stained for 10 - 15 minutes. The stained specimens were then washed for 30 seconds with distilled water and blotted dry on a filter paper. All specimens were stored in Petri dishes, lined with filter paper, until they were examined by transmission electron microscopy.

9. SUBCELLULAR FRACTIONATION

9.1 Homogenisation

Reagent:-

0.25M sucrose

Method:-

Male Wistar rats (200 - 250g), which had been fasted overnight, were killed by stunning. The liver was removed as quickly as possible and its weight recorded before it was cut into small pieces in sucrose on ice and then rinsed with two changes of sucrose. All subsequent operations in membrane preparation were carried out on ice unless otherwise stated.

The liver pieces were homogenised in sucrose (1ml per gram wet weight) by four passes with an Ilado (type X1020) tissue disperser set at 10,000 r.p.m. followed by four passes with a glass-teflon homogeniser at 600 r.p.m. The resultant crude homogenate was the starting material for all subcellular fractionation procedures unless otherwise indicated.

9.2 Preparation of mitochondria

Reagent:-

0.25M sucrose

Method:-

The rat liver crude homogenate, prepared as described in Section 9.1, was diluted by the addition of a further four volumes of 0.25M sucrose and centrifuged at 1500g for ten minutes at 2^{o}C to remove nuclei and cell debris. The pellet was discarded and the supernatant was

centrifuged at 10,000g for ten minutes at 2°C. The pellet was resuspended by mild glass-teflon homogenisation (four passes, 300 r.p.m.) in half the volume of 0.25M sucrose described above and washed by centrifugation. The washing procedure was repeated a second time and the resultant pellet was then used as soon as possible to assay the marker enzyme activities for outer mitochondrial membrane (monoamine oxidase), inner mitochondrial membrane (cytochrome oxidase) and lysosomal membrane (acid phosphatase) as described in Section 10 of METHODS.

9.3 Preparation of smooth endoplasmic reticulum-enriched fraction.

Reagents:-

0.25M, 0.6M and 1.3M sucrose

Method:-

The crude homogenate was centrifuged at 10,000g for 20 minutes at 2°C to pellet mitochondria, lysosomes, cell debris, etc. The pellet was discarded and the supernatant was centrifuged at 75,000g for 90 minutes at 2°C. The supernatant was discarded and the pellet, which contained microsomes, was carefully rinsed with 0.25M sucrose. The pellet was then resuspended until homogeneous, by glass-teflon homogenisation (600 r.p.m.), in a volume of sucrose equal to half that of the original crude homogenate. The resultant suspension contained a protein concentration between 15 and 20 $mg.ml^{-1}$. This suspension was then washed by centrifugation as described above.

The washed microsomes were resuspended by gentle glass-teflon homogenisation in 0.25M sucrose to a concentration of no more than 5 $mg.ml^{-1}$ so as to avoid aggregation (Depierre and Dallner, 1976). The suspension was carefully layered onto the top of a discontinuous sucrose density gradient which consisted of 4ml of 0.6M sucrose and 6ml of 1.3M sucrose in a 20ml polycarbonate centrifuge tube.

The gradient was then centrifuged at 100,000g for 2½ hours at 2°C in a MSE 3 x 20ml 'swinging-bucket' rotor. When centrifugation was completed the smooth membrane-enriched fraction, which accumulates above the interface between 0.6M and 1.3M sucrose (Depierre and Dallner, 1976), was carefully drawn off with a Pasteur pipette. The collected fraction was then made 0.25M with respect to sucrose, by the addition of distilled deionised water, and then centrifuged at 75,000g for one hour at 2°C.

The washed smooth membranes when required in micro-tubule-membrane interaction experiments (RESULTS Section 4) were resuspended, until homogenous, by gentle glass-teflon homogenisation in RB to a protein concentration of 4 - 5 $mg.ml^{-1}$. When the membranes were required for marker enzyme assays they were resuspended in 0.25M sucrose.

Rough endoplasmic reticulum-enriched membranes, which were pelleted after discontinuous sucrose density gradient centrifugation, were resuspended by gentle glass-teflon homogenisation in 0.25M sucrose and, along with resuspended smooth membranes and crude microsomes, were

assayed for glucose-6-phosphatase activity to measure the effectiveness of separation. The smooth membrane fraction was also assayed for several other marker enzyme activities, as described in METHODS Section 10, to assess the level of cross-contamination by other organelles.

It should be pointed out that the published method of smooth membrane preparation from which the above procedure was derived (Depierre and Dallner, 1976) involved the inclusion of 15mM $CsCl_2$ in the 0.6M and 1.3M sucrose gradient components. This is said to facilitate the aggregation of the rough microsomal membranes and the resultant increase in density increases their rate of sedimentation. Therefore, the separation of rough and smooth microsomal membranes is enhanced. However, when separations were carried out using $CsCl_2$ in this project, the smooth membrane yields were much too low (0.2 - 0.3mg protein per gram original wet weight) to carry out experiments using fresh membranes on the scale required.

It was decided to exclude $CsCl_2$ from the sucrose gradient to increase membrane yields at the risk of higher levels of contamination by rough membranes. The resultant yields were within the range 1 - 2mg protein per gram wet weight tissue. Marker enzyme assays and electron microscopy (RESULTS Section 4) showed that the major component of the fraction collected at the 0.6M - 1.3M sucrose interface was smooth endoplasmic reticulum (SER).

9.4 Preparation of golgi apparatus

Reagents:-

(a) Homogenisation medium - 37.5mM Tris
0.5M sucrose
1% dextran (average molecular weight, 170,000).
pH 6.4, adjusted with 2-(N-morpholino) ethane sulphonic acid (MES).

(b) 1.2M sucrose.

Method:-

The procedure used was based on the method described by Morré, Yunghans, Vigil and Keenan (1974) with slight modifications. Rat liver (10 grams in 25ml of homogenisation medium) was homogenised in an Ilado (type X1020) tissue disperser at 10,000 r.p.m. for 40 seconds on ice. The resultant homogenate was then centrifuged at 5,000g for 15 minutes at 2°C. The supernatant was carefully removed and the loose yellow-brown upper portion of the pellet was transferred to another tube with the aid of a large-bore Pasteur pipette. This material was then resuspended by gentle swirling to a total volume of 6ml with the supernatant fluid described above.

The suspension was then layered onto a sucrose cushion, which consisted of 10ml of 1.2M sucrose, in a 20ml polycarbonate centrifuge tube. This was then centrifuged at 100,000g for 20 minutes at 2°C in a MSE 3 x 20ml swinging-bucket rotor. A band, which was enriched in

golgi apparatus membranes (Morré et al., 1974), collected above the 1.2M sucrose interface and was removed with a Pasteur pipette. Care was taken not to remove any of the 1.2M sucrose cushion. This fraction was then made up to 5ml by the addition of homogenisation medium, and centrifuged at 5,000g for 15 minutes at 2°C. The resultant pellet was resuspended to a concentration of about 5mg.ml^{-1} protein in 0.25M sucrose and used as a 100% reference for the golgi apparatus marker enzyme thiamine pyrophosphatase (METHODS 10.3).

10. MEMBRANE MARKER ENZYME ASSAYS

10.1 Glucose-6-phosphatase

The procedure used was based on the method of Morré (1971).

Reagents:-

(a) 55mM TRIS, pH 6.6

(b) 11mM glucose-6-phosphate

(c) 11mM 2-mercaptoethanol

(d) 10% w/v trichloroacetic acid (TCA)

(e) 2.5% w/v ammonium molybdate in 5N H_2SO_4

(f) Aminonaptholsulphonic acid reagent (ANSA)

Method:-

Tubes containing 0.9ml of TRIS, glucose-6-phosphate and mercaptoethanol (at the final concentrations described above) were equilibrated at 37°C for 5 minutes. The reaction was initiated by the addition of 0.1ml (0.2 - 0.5mg protein) of membrane preparation resuspended in 0.25M sucrose, and was allowed to proceed for 15 minutes

to release inorganic phosphate within the range 0.1 - 1.0 µmoles. Termination of the reaction was achieved by the addition of 1ml of 10% TCA after which the mixture was clarified by centrifugation (2,000 r.p.m. for 15 - 20 minutes in a MSE bench centrifuge).

A phosphate determination was then carried out on 1ml of the clear supernatant which was vortex-mixed after the addition of 1ml of ammonium molybdate-H_2SO_4 followed by 0.2ml of ANSA. The volume was then made up to 5ml with distilled deionised water and vortex-mixed. After 20 minutes at room temperature the absorbance was measured at 660nm in a Cecil single-beam spectrophotometer. A calibration curve was obtained from a series of standard samples which contained 0 - 100µg KH_2PO_4 and were treated, as described above, in parallel.

10.2 5'-nucleotidase

The procedure used was exactly as described by Morré (1971).

Reagents:-

(a) 55mM TRIS, pH adjusted to 8.5 with HCl.

(b) 11mM adenosine 5'-monophosphate (AMP)

(c) 5.5mM $MgCl_2$

(d) 10% w/v TCA

(e) 2.5% w/v ammonium molybdate in 5N H_2SO_4

(f) ANSA

Method:-

Test tubes containing 0.9ml total volume of a mixture of 55mM TRIS, 11mM AMP, 5.5mM $MgCl_2$ were equilibrated at 37°C for 5 minutes. The reaction was initiated by the

addition of 0.1ml (0.2 - 0.5mg protein) of microsomal or endoplasmic reticulum membranes (prepared as described in METHODS 9.3) resuspended in 0.25M sucrose. After 15 minutes the reaction was terminated by the addition of 1ml of 10% TCA and the samples clarified by centrifugation (2,000 r.p.m. for 15 - 20 minutes in a MSE bench centrifuge).

An inorganic phosphate determination was then carried out on 1 ml of the clear supernatant. This was vortex-mixed with 1 ml of 2.5% ammonium molybdate in 5N H_2SO_4 followed by 0.2ml of ANSA. The volume was made up to 5ml with the addition of distilled water, vortex-mixed, and the absorbance read, after 20 minutes at room temperature, at 660nm in a Cecil single-beam spectrophotometer. A calibration curve was obtained from a series of standards containing 0 - 100µg KH_2PO_4 which were assayed in parallel.

10.3 Thiamine pyrophosphatase

The procedure adopted was that described by Allen and Slater (1961).

Reagents:-

(a) Substrate - 33mM sodium barbital
15mM $CaCl_2$
3.3mM thiamine pyrophosphate hydrochloride
pH 8.0, adjusted with NaOH.

(b) 10% w/v TCA

Method:-

The reaction was initiated by the addition of 0.1ml of golgi apparatus or smooth endoplasmic reticulum membranes (prepared as shown in METHODS 9.3), resuspended in 0.25M sucrose, to 2.9ml of substrate. The reaction was terminated after 20 minutes at 37°C by the addition of 1ml of 10% TCA. The samples were then clarified by centrifugation for 15 - 20 minutes at 2,000 r.p.m. in an MSE bench centrifuge. Then 2ml of the clear supernatant were taken for inorganic phosphate assay as described in Section 10.1.

10.4 Monoamine oxidase

The procedure adopted was based on the method of Robinson, Lovenberg, Keiser and Sjoerdsma (1967) with several modifications.

Reagents:-

(a) 0.2M Na_2HPO_4, pH 7.2

(b) 7-^{14}C tyramine hydrochloride - to use in assays, 1ml (50 µCi) of radio-labelled isotope (specific activity 50 mCi.mmol^{-1}) was diluted with 28.6mg of unlabelled tyramine and 15.6ml of distilled water.

(c) 6N HCl

(d) Benzene : ethyl acetate (1:1, by volume)

(e) Triton scintillation fluid - as described in METHODS 3.2.

Method:-

Each assay tube (glass-stoppered vial) contained 750 µl of Na_2HPO_4 and 150µl of membrane extract (0.2 - 0.5 mg protein). Mitochondrial or smooth endoplasmic reticulum membrane extracts, resuspended in 0.25M sucrose after preparation as described in METHODS 9.2 and 9.3, were frozen (-20°C) and thawed twice before use.

To the control samples, 100µl of 6N HCl were added to inactivate enzyme activity.

Then 100µl of ^{14}C-tyramine, diluted as described above, were added to all assay tubes and mixed with a vortex mixer. The samples were then incubated for one hour in a shaking water bath at 37°C, after which the reaction in the test samples was terminated by the addition of 100µl of 6N HCl.

Deaminated products of the reaction were extracted by the addition of 2ml of benzene:ethyl acetate after which the tubes were agitated for ten minutes on a motor-driven test tube shaker. The assay tubes were then left, standing upright, until the contents separated into two phases without interfacial fluff. Then 1ml of the upper phase from each extraction was transferred into individual scintillation vials containing 10ml of Triton scintillation fluid. In addition, a sample containing 0.1ml of labelled substrate was also added to 10ml scintillation fluid. All samples were counted for ten minutes in a Kontron (Intertechnique) liquid scintillation counter. The specific activity, in µmoles substrate hydrolysed per hour per mg protein, was

calculated from the equation:-

$$\frac{\text{(c.p.m. of sample - c.p.m. of blank) x 2}}{\text{c.p.m. of 0.1ml tyramine substrate x protein content of sample}}$$

10.5 Cytochrome oxidase

Reagents:-

(a) 0.1M Sodium phosphate (NaH_2PO_4) pH 7.0

(b) 1% w/v cytochrome c in 0.01M sodium phosphate

(c) 0.1M potassium ferricyanide

(d) Sodium dithionite crystals

Method:-

The procedure was based on the method described by Wharton and Tzalgoloff (1967). Cytochrome c was chemically reduced before each assay by the addition of a few crystals of sodium dithionite. Excess dithionite was removed by bubbling air through the reduced solution (Hodges and Leonard, 1974).

To each of two glass cuvettes, 0.1ml of 0.1M potassium phosphate, 0.7ml of 1% cytochrome c and 0.83ml of distilled water were added. The cuvettes were then equilibrated in a water bath for five minutes at 38°C. One of these samples was used as reference, whereas 10µl of enzyme extract (mitochondria or SER prepared as described in METHODS 9.2 and 9.3), containing 10 - 100µg protein, were added to the other cuvette to initiate the reaction. The fall in absorbance at 550nm was monitored in a Pye Unicam SP30 spectrophotometer. When the rate

of fall in absorbance tailed off (3 - 4 minutes) the reference cuvette contents were completely oxidised by the addition of 10µl of potassium ferricyanide.

The first order velocity constant (K) was calculated from the equation:-

$$K = 2.3 \log \frac{\text{absorbance at time zero}}{\text{absorbance after one minute}}$$

Specific activity was then calculated from the known concentration of cytochrome c and membrane protein in the assay mixture and the estimated velocity constant:-

$$\frac{60K \times \text{concentration cytochrome c}}{\text{concentration of membrane protein}}$$

µmol. per hour per mg. protein

10.6 Acid phosphatase

The procedure was exactly as that described by Leonard and Hodges (1974).

Reagents:-

(a) Substrate - 37.5mM TRIS
55.5mM KCl
3.33mM p-nitrophenyl phosphate
1.67mM $MgSO_4$
pH 5.5, adjusted with MES

(b) 1% w/v ammonium molybdate in 2N sulphuric acid

(c) ANSA

Method:-

The reaction was started by the addition of 0.1ml of the membrane fraction (crude homogenate or SER

prepared as in METHODS 9.1 and 9.3), containing 10 - 50µg protein, to 0.9ml of substrate which had been equilibrated for five minutes at 38°C. The reaction was allowed to proceed for 10 - 45 minutes (to check linearity with time and protein concentration). The reaction was terminated by the addition of 2ml of ice-cold 1% ammonium molybdate in 2N sulphuric acid and the assay tubes placed on ice.

For the determination of inorganic phosphate, 0.4ml of ANSA was added and thoroughly mixed. Assay tubes were then incubated at room temperature for 35 minutes. Then the absorbance of the samples was measured at 660nm in a Pye Unicam SP30 or a Cecil single-beam spectrophotometer. A calibration curve was obtained from a series of standards containing 0 - 100µg of KH_2PO_4 which were assayed in parallel.

11. PROTEIN ASSAY

Protein assays were carried out by a modification of the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard.

Reagents:-

(a) Reagent A - 0.5% w/v copper sulphate in 1% w/v sodium-potassium tartrate

(b) Reagent B - 2% w/v Na_2CO_3 in 0.1M NaOH

(c) Reagent C - 1ml of reagent A mixed with 50ml of reagent B immediately prior to the assay

(d) Folin-Ciocalteau reagent - diluted 1:1 with distilled water

(e) Bovine serum albumin

(BSA) - Stock solution of 100µg.ml^{-1}

Method:-

Five ml of reagent C was vortex-mixed with 1ml of protein extract diluted with distilled water to a concentration of 20 - 70µg.ml^{-1}. The reactants were incubated at room temperature for ten minutes after which 0.5ml of diluted Folin-Ciocalteau reagent was added and vortex-mixed. The absorbance of each sample was read, after about 30 minutes at room temperature, in a Cecil single-beam spectrophotometer at 750nm. A calibration curve was obtained from a series of standards containing BSA (0 - 80µg ml^{-1}) which were assayed in parallel.

Calibration curves were linear over this range of BSA concentration at all dilutions of PIPES, phosphocellulose column buffer or sucrose included in the assay at any time throughout this project. It was observed, however, that different dilutions of these buffers resulted in a different gradient for the calibration curve and that the calibration curve departed from linearity at slightly higher BSA concentrations. It was, therefore, important to ensure that the composition of test samples and BSA standards were exactly the same with respect to the buffer used in the previous preparation or experiment.

RESULTS

1. BIOCHEMICAL ANALYSIS OF MICROTUBULE ASSEMBLY IN VITRO

Microtubule proteins for all experiments presented in this thesis were prepared from pig brain by temperature-dependent recyclization as described in Methods 2. It was important to establish that the buffer conditions used for microtubule preparation, and subsequent experiments with the purified microtubule protein, were optimal and that the protein composition and biochemical properties of microtubule preparations were comparable to those described by other workers.

1.1 Effects of GTP, cations and colchicine on microtubule assembly.

Microtubule assembly was not detectable in the absence of added GTP. There was a gradual increase in the extent of microtubule assembly, as determined turbidimetrically (Methods 3.1), up to a concentration of 0.5mM added GTP (Figure 1a). Higher concentrations of GTP caused no further enhancement of assembly.

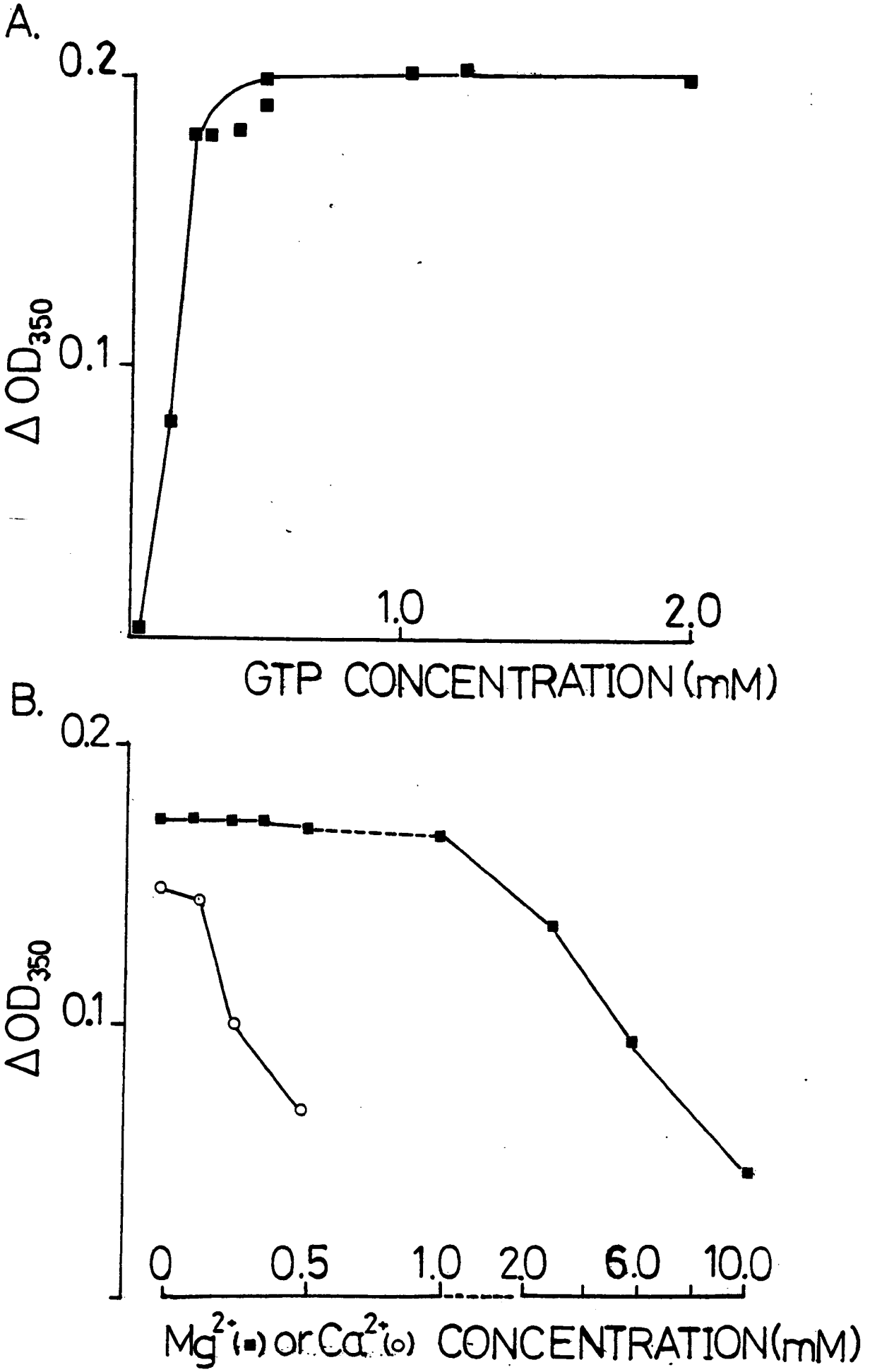
There was an absolute requirement for Mg^{2+} ions which was indicated by the complete inhibition of microtubule assembly in the presence of EDTA to chelate Mg^{2+}. This effect could be reversed on the addition of an excess of Mg^{2+} (not shown). Microtubule assembly was not affected by the addition of 0 - 0.5mM $MgSO_4$ under normal buffer conditions which included 1mM EGTA (which chelates only Ca^{2+}) (Figure 1b), but higher concentrations reduced the extent of microtubule assembly. It was decided to include Mg^{2+} at

FIGURE 1

The effects of GTP and divalent cations on the extent of microtubule assembly.

(a) One-cycle-purified microtubule proteins, resuspended in RB at pH 6.8 to a concentration of 2.4mg.ml^{-1}, were incubated for 20 minutes at 37°C after the addition of various concentrations of GTP, as indicated. Microtubule assembly was calculated as the total change in optical density measured at 350nm (ΔOD_{350}). Each data point represents a mean of duplicate samples. The results were combined from two independent experiments, run at the same protein concentration.

(b) One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2 - 3 mg.ml^{-1}, were incubated for 20 minutes at 37°C after the addition of 0.5mM GTP. $MgSO_4$ or $CaCl_2$ were included at the final concentrations indicated. In the Ca^{2+} experiment EGTA, which chelates Ca^{2+}, was omitted from the buffer. The extent of microtubule assembly was determined as the change in optical density at 350nm (ΔOD_{350}) over the incubation period. Each data point represents a mean of duplicate samples. Results are shown from three separate experiments which included 0 - 0.5mM Mg^{2+}, 1 - 10mM Mg^{2+}(■) and 0 - 0.5mM Ca^{2+}(o) as indicated. A duplicate experiment, at a higher microtubule protein concentration, showed similar results.


A.
0.2
0.1
ΔOD350
1.0
2.0
GTP CONCENTRATION (mM)
B.
0.2
0.1
ΔOD350
0
0.5
1.0
2.0
6.0
10.0
Mg2+(■) or Ca2+(○) CONCENTRATION(mM)

a concentration of 0.5mM in all subsequent experiments and preparations of microtubules to ensure the presence of sufficient free Mg^{2+} to maintain optimal conditions for microtubule assembly.

In contrast, preincubation of microtubule proteins with Ca^{2+} ions up to a concentration of only 0.5mM resulted in approximately 60% inhibition of microtubule assembly (Figure 1b). Such effects were not reversible on the addition of excess EGTA which suggested that the Ca^{2+} may have activated an inhibitory enzyme, possibly a protease such as that described by Sandoval and Weber (1978). For this reason it was decided to include 1mM EGTA in all subsequent assays and preparations to chelate any traces of free Ca^{2+}.

The addition of 2mM Ca^{2+} to completely polymerized microtubules resulted in an instantaneous decrease in turbidity (Figure 2a). Samples which were examined in an electron microscope indicated the formation of ring or disc-shaped structures after the addition of Ca^{2+}, and the total destruction of microtubules (Figures 2b and 2d). These structures were similar to those described by other workers as structural intermediates in microtubule assembly (Kirschner et al., 1975).

Another microtubule inhibitor namely colchicine was also studied in the same experiment. When added to microtubule proteins at 37°C prior to the addition of GTP 10μM colchicine resulted in almost complete inhibition of turbidity development (Figure 2a).

Samples examined by electron microscopy indicated the presence of only a few relatively short microtubules

FIGURE 2

Effects of Ca^{2+} and colchicine on microtubule assembly

2a. One-cycle-purified microtubule proteins, resuspended in RB at pH 6.8, at a concentration of 2.4mg.ml^{-1} were incubated at 37^{o}C in the presence of 0.5mM GTP for 15 minutes. In the spectrophotometer recordings shown, one specimen included 10μM colchicine (trace 1) and in the other (trace 2) 2mM Ca^{2+} was added after 15 minutes incubation (↓). Specimens were prepared for electron microscopy, by direct negative staining with 1% uranyl acetate, where indicated (b, c or d).

2b. Magnification x 20,000; polymerized microtubules after plateau reached on spectrophotometer recording.

2c. Magnification x 20,000; microtubule proteins incubated in the presence of 10μM colchicine. Very few, short microtubules were observed.

2d. Magnification x 40,000; after the addition of 2mM Ca^{2+} to preformed microtubules, only disc-like structures were observed.

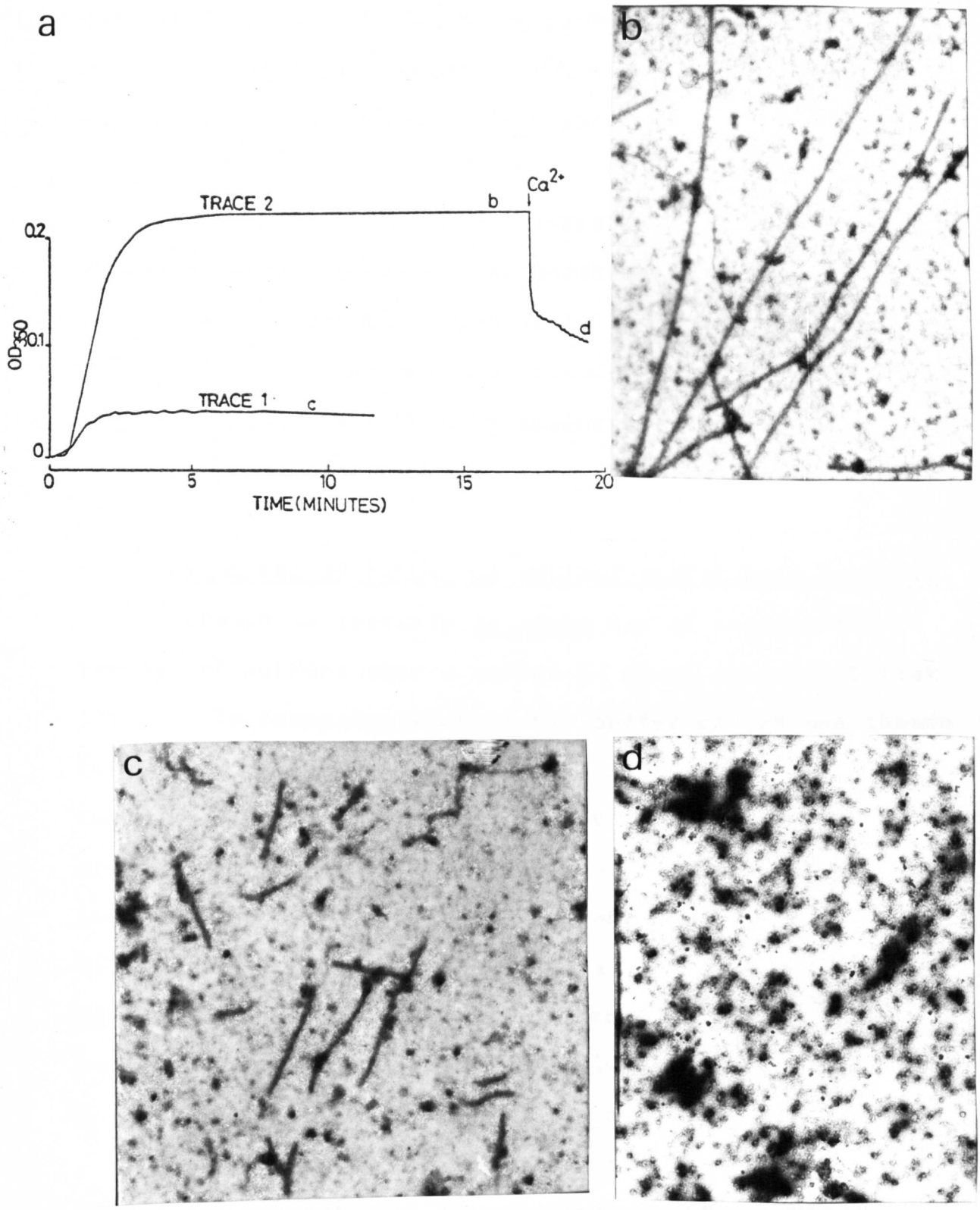


FIGURE 2

(Figure 2c). In replicate experiments, where lower protein concentrations were used, turbidity development was totally inhibited in the presence of colchicine (not shown).

Thus, the instantaneous reversal of turbidity of assembled microtubules on the addition of Ca^{2+} and the inhibition of assembly by antimitotic drugs could be used in future experiments as chemical markers to assess the reliability of turbidity measurements as a reflection of microtubule assembly.

1.2 The useful pH range for optimal microtubule assembly.

Microtubule assembly *in vitro* can be supported in a variety of buffers over a narrow pH range around 6.8 (Sakai, 1980). To determine whether the buffer conditions chosen for the experiments in this thesis were within an optimal range an experiment was performed to investigate the reversibility of turbidity charge of microtubule proteins, incubated at 37°C in the presence of GTP at various pH values by a shift in temperature to 4°C. The use of temperature-reversal was based on the observation that cold-labile microtubules will depolymerize completely over a period of time at 4°C (Gaskin *et al.*, 1974).

The data in Table 1 show that the temperature-reversal effect was highest over the pH range 6.6 - 7.0 and a slight peak was observed at pH 6.8. Reversal was markedly lower either side of this pH range.

At relatively low pH values (e.g., 6.1) there was an instantaneous rise in turbidity which continued to rise for

TABLE 1

Effects of pH on microtubule assembly.

One-cycle-purified microtubule proteins resuspended in RB at various pH values, as indicated, to a concentration of approximately 3mg.ml^{-1} were incubated at 37^{o}C for 40 minutes after the addition of 0.5mM GTP. The total increase in optical density at 350nm was noted and then the samples were incubated on ice for 40 minutes. The resultant decrease in optical density was then noted. The results are expressed as a percentage of the optical density change (%ΔOD_{350}), after incubation at 37^{o}C, which was reversed at low temperature and represent the mean $\pm$ mean difference for duplicate readings. Similar results were obtained in a duplicate experiment at a higher protein concentration.

FIGURE 3.

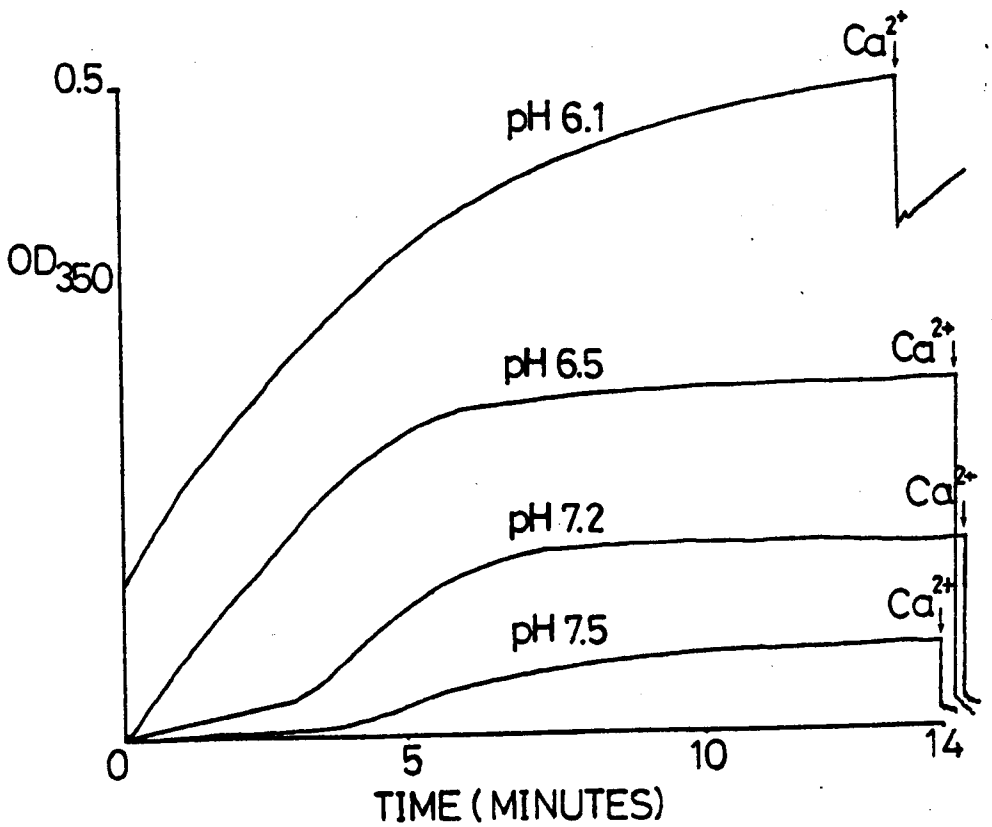
Effects of pH on turbidity development of microtubule proteins

One-cycle-purified microtubule proteins resuspended in RB, at various pH values as indicated, to a concentration of 2.6mg.ml^{-1} were incubated at 37^{o}C for 15 minutes after the addition of 0.5mM GTP. Shown are tracings of typical spectrophotometer recordings (one of two experiments) of change in optical density at 350nm (OD_{350}). Arrows indicate points at which 10mM Ca^{2+} was added.

TABLE 1

pH	%ΔOD reversible on cooling at 4°C
6.1	65.0 ± 5.0
6.6	79.7 ± 9.0
6.7	75.8 ± 3.6
6.8	83.0 ± 7.0
6.9	82.0 ± 2.7
7.0	81.0 ± 2.9
7.9	74.1 ± 2.7

FIGURE 3



at least one hour and was not reversible on the addition of 10 mM Ca^{2+} (Figure 3). In contrast the turbidity development at pH 6.6 - 7.0 produced a characteristic plateau-type curve, after an initial short lag period, over a period of only a few minutes. In such cases turbidity development was reversible with Ca^{2+}. Higher pH produced lower tubidity development. Although analysis of samples by electron microscopy confirmed the presence of microtubules in all samples (not shown), the instantaneous rise in turbidity at pH 6.1 suggested that some other protein aggregation process may be involved. It was decided to use pH 6.8 in all future experiments as it appeared to promote optimal microtubule assembly in this system. Turbidity development under these conditions is from this point onwards assumed to be a direct reflection of microtubule assembly under the established optimal buffer conditions.

1.3 The effects of total protein concentration and temperature on microtubule assembly.

From the results shown in Figure 4 it can be seen that both the initial rate and extent of microtubule assembly increased with protein concentration and the initial lag period, observed before commencement of microtubule assembly, became shorter. Microtubule assembly was not detectable by turbidity measurement at protein concentrations below $0.6 mg.ml^{-1}$.

Optimal microtubule assembly was achieved over the temperature range of 30 - 37°C with a slight peak at 37°C

FIGURE 4

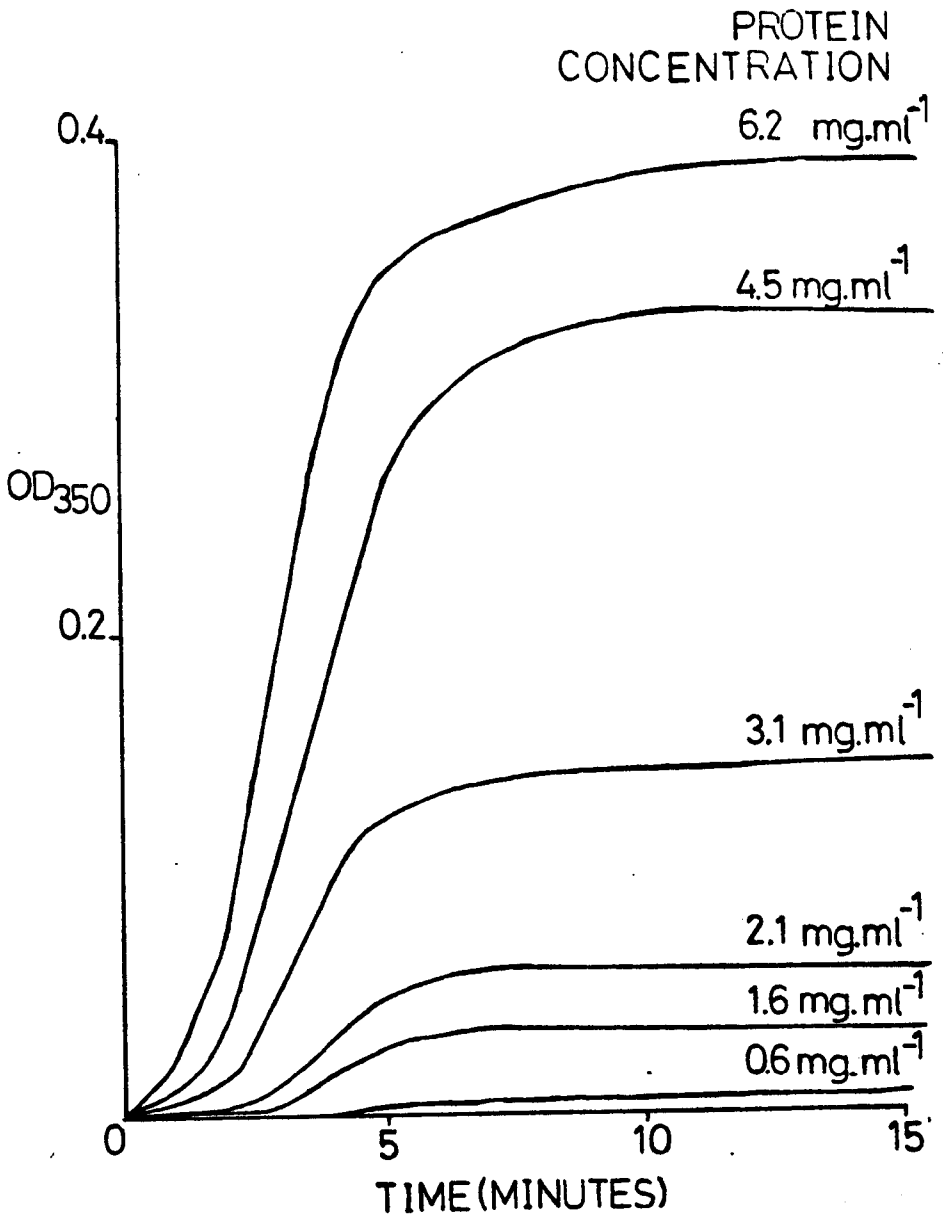
Effects of protein concentration on microtubule assembly.

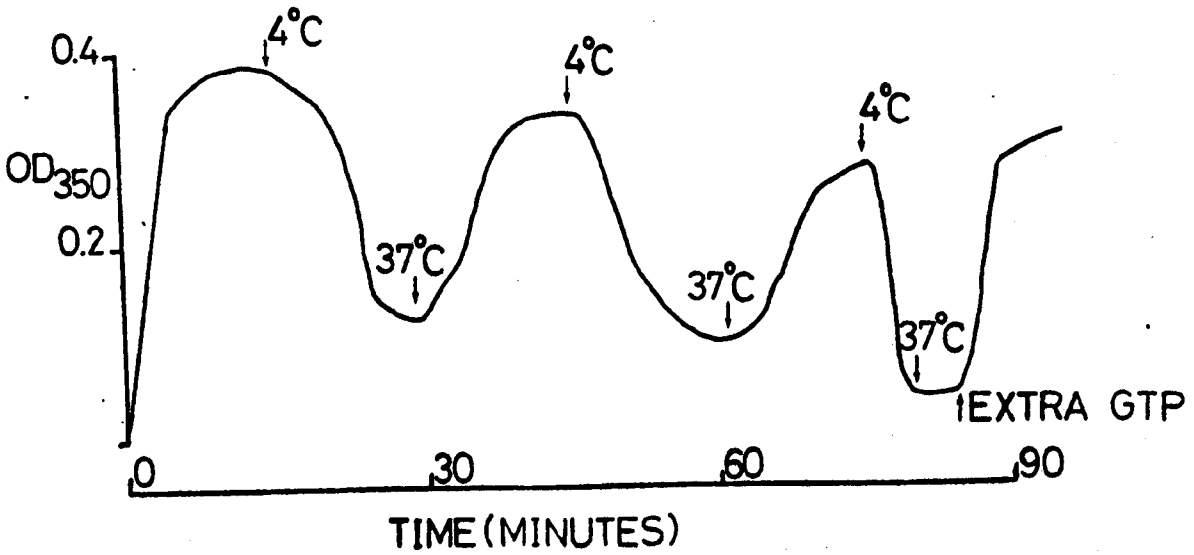
One-cycle-purified microtubule proteins resuspended in RB at pH 6.8, at various protein concentrations as indicated, were incubated for 15 minutes at 37°C after the addition of 0.5mM GTP. Shown are typical spectrophotometer recordings (from one of two experiments) of change in optical density at 350nm (OD_{350}).

FIGURE 5.

Temperature-reversible microtubule assembly-disassembly.

One-cycle-purified microtubule proteins, resuspended in RB at pH 6.8 and at a concentration of 6mg.ml^{-1}, were incubated in the presence of 0.5mM GTP for a period of approximately 90 minutes. During this period microtubule assembly was initiated at 37°C and disassembly at 4°C, in a spectrophotometer fitted with a thermostatted cell-housing, as indicated by downward arrows. Shown is a tracing of a typical spectrophotometer recording of change in optical density at 350nm (one from two experiments). After three cycles of temperature-reversible assembly-disassembly, a further round of assembly at 37°C could only be induced if extra GTP (0.5mM, upward arrow) was added.


PROTEIN
CONCENTRATION
6.2 mg.ml-1
4.5 mg.ml-1
3.1 mg.ml-1
2.1 mg.ml-1
1.6 mg.ml-1
0.6 mg.ml-1
0.4
OD350
0.2
0
5
10
15
TIME (MINUTES)


0.4
OD350
0.2
4°C
4°C
4°C
37°C
37°C
37°C
EXTRA GTP
0
30
60
90
TIME (MINUTES)

(data not shown). Subsequently all experiments and preparations were carried out at 37°C for comparison purposes with other workers (Larsson et al., 1976; Borisy et al., 1974). The reversibility of microtubule assembly by temperature shift between 37°C and 4°C under optimal buffer conditions, is illustrated in Figure 5. It was shown that 0.5mM GTP was able to support three cycles of temperature-reversible assembly-disassembly after which the addition of extra GTP was required to produce further microtubule assembly. A similar result was obtained by Kirazov and Lagnado (1977) after three cycles of assembly-disassembly in chick brain microtubule preparations.

1.4 Electrophoretic analysis of microtubule proteins.

As discussed in the Introduction, microtubule proteins isolated by temperature-dependent recyclization are known to be composed of mainly tubulin along with a variety of accessory proteins. The relative amount of accessory proteins present appears to depend on the buffer conditions chosen for microtubule preparation. Another factor involved could be the release of proteolytic enzymes after death of the animal or during subsequent fractionation of the tissue used. This factor could be particularly important where a visit to the abattoir is necessary.

Precautions used to reduce proteolytic activity involved in the preparations used in this thesis included the immediate removal of superficial blood vessels and cutting up the brain into small pieces in ice-cold buffer. These operations were carried out at the abattoir usually

within one hour of slaughter. However, it was still necessary to establish that the electrophoretic pattern of the microtubule proteins used in these experiments was comparable to those described by other workers and reproducible from one preparation to another.

From the electrophoretic patterns shown in Figure 6, 20 - 30 polypeptide bands can be seen on overloaded gels. When 2 - 10µg total protein was applied to the gel it was shown that the major components were tubulin (apparent molecular weight 55,000) some high molecular weight doublet species (>200,000) which correspond in molecular weight to MAPs (Murphy and Borisy, 1975) and a group of 4 or 5 polypeptides over the molecular weight range 60,000 - 70,000 which may correspond to tau factor (Cleveland et al., 1977).

Densitometric analysis of gels containing 2 - 10µg microtubule protein showed that the tubulin bands accounted for 51 - 56% of the total peak area at one cycle of purification (four gels) and 70 - 75% at three cycles of purification (three gels). It should be noted, however, that this type of quantitative analysis assumes that the 55,000 molecular weight polypeptide contains tubulin only, and that all proteins stain in a quantitative manner with coomassie blue reagent.

FIGURE 6.

Electrophoretic analysis of microtubule proteins.

One-cycle-purified microtubule proteins were resuspended in RB to a concentration of 2 - 3mg.ml^{-1} and aliquots were freeze-dried. Freeze-dried samples were then resuspended in an equal volume of electrophoresis sample buffer, boiled for two minutes, and electrophoresed on a 4 - 15% polyacrylamide gradient gel with a 4% stacking gel. Protein standards were dissolved directly in sample buffer to a concentration of 1 mg/ml^{-1}, treated as described above, and then electrophoresed in parallel.

Gel A is an overloaded gel which contained approximately 70µg protein. Gel B, which contained 8µg protein, showed the major constituents of microtubule preparations to have molecular weights which correspond to tubulin (50,000), tau polypeptides (60,000 - 70,000) and MAPs 1 and 2 (>200,000). Gel C contained 20µg each of the low molecular weight protein standards cytochrome C (11,700), gamma globulins (25,000 and 50,000) and transferrin (70,000). Gel D contained 20µg of cross-linked haemocyanin, a high molecular weight standard over the range 53,000 (monomer) to 265,000 (tetramer).

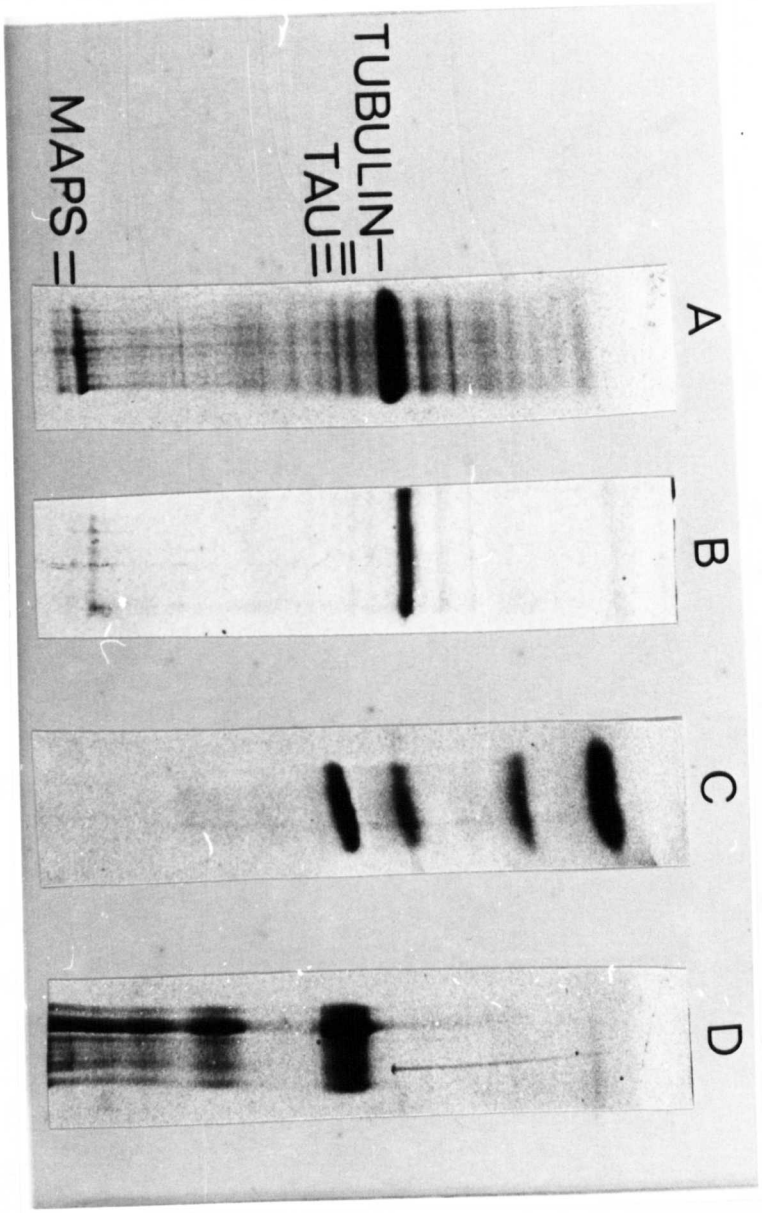


FIGURE 6.

2. ANALYSIS OF MICROTUBULE-ASSOCIATED PHOSPHOLIPIDS

The aim of the experiments described in this Section was to extract and identify the phospholipids associated with pig brain microtubules, to quantify their association with highly-purified microtubule protein and to determine whether specific protein-phospholipid associations occurred.

2.1 Identification of phospholipids associated with one-cycle-purified microtubule pellets.

Phospholipids associated with microtubule pellets after one cycle of purification were identified after tlc (Figure 7) by comparison of their R_f values (Table 2) with those of commercial lipid standards (Table 3) and by their reaction with specific spray reagents (Table 4). There were thirteen components detectable on charred chromatograms of one-cycle-purified microtubule-associated phospholipids (Figure 7). However, spots numbered 8 and 13 were shown to be breakdown products which appeared on a chromatogram of a mixture of phospholipid standards (Figure 8). In addition, it was shown that these components were not artifacts of the phospholipid extraction procedure nor solvent impurities as they were absent from chromatograms run of (i) a chloroform:methanol extract of RB prepared in the absence of microtubule protein and (ii) of solvents alone (not shown).

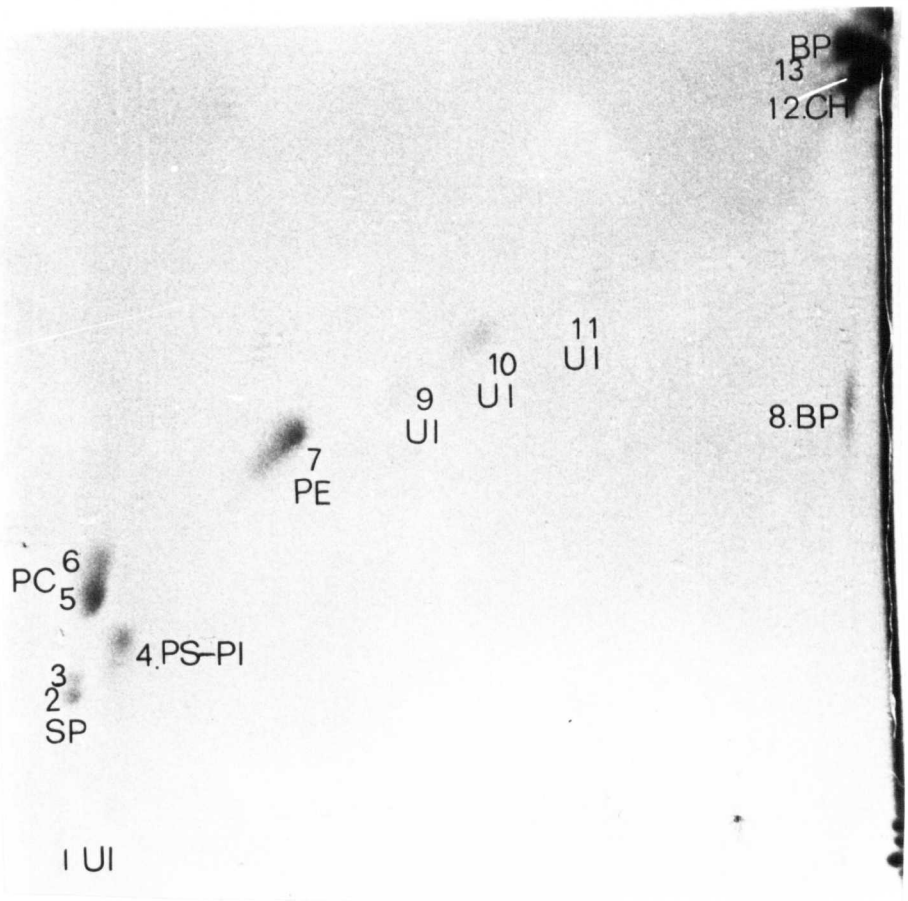
Spot 1 (Figure 7) was not identifiable at this stage as it did not correspond in R_f to any of the commercial standards used, nor did it react with a specific spray

FIGURE 7

Two-dimensional tlc of one-cycle-purified microtubule-associated phospholipids.

Phospholipids were extracted from microtubules, pelleted by centrifugation after one cycle of temperature-dependent recyclization, in chloroform: methanol (2:1) by the method of Folch et al., (1957). Shown is a photograph of a typical plate (one from a series of twelve run in parallel) where visualisation was by charring. Chromatogram components were identified, as indicated, by comparison with standards and reaction with specific spray reagents. The abbreviations used are PC (phosphatidyl choline), PE (phosphatidyl ethanolamine), SP (sphingomyelin) PS (phosphatidyl serine), PI (phosphatidyl inositol), CH (cholesterol), UI (unidentified) and BP (breakdown product).

The numbers of chromatogram components correspond to those in the R_f table (Figure 8). The inset (bottom right) shows a chromatogram of phospholipid and cholesterol standards which was run in parallel.


BP
13
12.CH
11
UI
10
UI
9
UI
8.BP
7
PE
PC
6
5
4.PS-PI
3
2
SP
1 UI

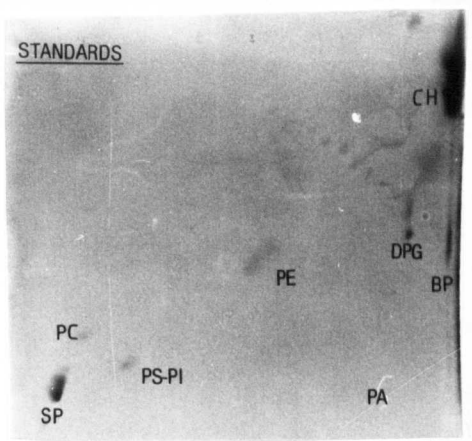

STANDARDS
CH
DPG
PE
BP
PC
PS-PI
PA
SP

FIGURE 7

TABLE 2

Relative mobilities of microtubule-associated phospholipids and cholesterol.

Microtubules were pelleted by centrifugation after one, two or three cycles of temperature-dependent recyclization. Phospholipids were extracted from four 2.5mg samples at each cycle of purification and separated in parallel by two-dimensional tlc. After visualisation by charring, relative mobilities (R_f) were calculated for each chromatogram component numbered 1 - 13, as indicated. Results are expressed as mean $\pm$ S.D. of twelve observations (from twelve separate chromatograms) except in the case of trace components 9, 10 and 11, which were only detected at one cycle of microtubule purification on 2, 4 and 4 chromatograms respectively.

TABLE 2

SPOT NUMBER (As in Fig.7)	R_f FIRST DIMENSION	R_f SECOND DIMENSION
1	zero	zero
2	.193 ± .016	.030 ± .008
3	.214 ± .016	.034 ± .008
4	.261 ± .012	.122 ± .056
5	.307 ± .020	.074 ± .028
6	.343 ± .020	.086 ± .032
7	.519 ± .036	.384 ± .100
8	.580 ± .044	.955 ± .016
9	.6	.46
10	.665 ± .256	.522 ± .100
11	.759 ± .244	.663 ± .120
12	.895 ± .265	.954 ± .016
13	.975 ± .032	.973 ± .052

TABLE 3

Relative mobilities of phospholipid and cholesterol standards.

Duplicates of standard mixtures which contained 2.5, 5, 10, 15, 20 or 25µg each of phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl inositol (PI), sphingomyelin (SP), cardiolipin (DPG), phosphatidic acid (PA) and cholesterol (CH) were separated by two-dimensional tlc. Chromatograms were visualised by charring and R_f values were calculated for each component, in both dimensions, from each of the twelve chromatograms run in parallel. Results are expressed as mean $R_f \pm$ S.D. of ten observations.

PE and SP standards migrated as double spots, which indicated heterogeneity, and so both R_f values designated (1) and (2) are presented. PS and PI standards comigrated and so only one R_f (PS - PI) is presented in this case.

TABLE 3

CHROMATOGRAM COMPONENT	FIRST DIMENSION	SECOND DIMENSION
SP(1)	.207 ± .016	.035 ± .006
SP(2)	.235 ± .016	.041 ± .006
PS - PI	.288 ± .019	.139 ± .006
PC	.358 ± .025	.097 ± .019
PA	.260 ± .016	.583 ± .073
PE(1)	.554 ± .047	.493 ± .047
PE(2)	.592 ± .051	.527 ± .047
DPG	.585 ± .076	.672 ± .035
CH	.895 ± .013	.957 ± .010

TABLE 4

Identification of chromatogram components with specific spray reagents.

Microtubules were pelleted by centrifugation after one cycle of temperature-dependent recyclization. Phospholipids were extracted from 10 - 15mg of microtubule protein and separated by two-dimensional tlc. Chromatogram components were visualised by iodine vapour and lightly marked in pencil. The iodine was allowed to fade before the application of the appropriate specific spray reagent as indicated. Standard phospholipids were spotted onto tlc plates at a number of concentrations to estimate cross-reactions and sensitivity to the spray reagents, as shown in Methods. Where indicated by 'S' the chromatogram components did not react consistently with molybdenum spray reagent, presumably due to their low concentrations.

TABLE 4

REAGENT	CHROMATOGRAM COMPONENT (numbered as in Figure 7 and Table 2).												
	1	2	3	4	5	6	7	8	9	10	11	12	13
iodine	+	+	+	+	+	+	+	+	+	+	+	+	+
charring	+	+	+	+	+	+	+	+	+	+	+	+	+
molybdenum	-	+	+	+	+	+	+	+	S	S	S	-	-
ninhydrin	-	-	-	+	-	-	+	-	-	-	-	-	-
Dragendorff	-	-	-	+	+	-	-	-	-	-	-	-	-
benzidene	-	+	+	-	+	+	+	-	-	-	-	-	-
glacial acetic/H_2SO_4	—	—	—	—	—	—	—	—	—	—	—	+	-

FIGURE 8

Two-dimensional tlc of phospholipid and cholesterol standards.

Shown is a schematic representation of a mixture of 20µg each of phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl inositol (PI), sphingomyelin (SP), phosphatidic acid (PA) and cholesterol (CH) separated by two-dimensional tlc. The chromatogram shown (oneof two) was visualised by iodine and then traced. The shaded area of the PS - PI component represents the portion of this component (when stained with iodine) which reacted with ninhydrin.

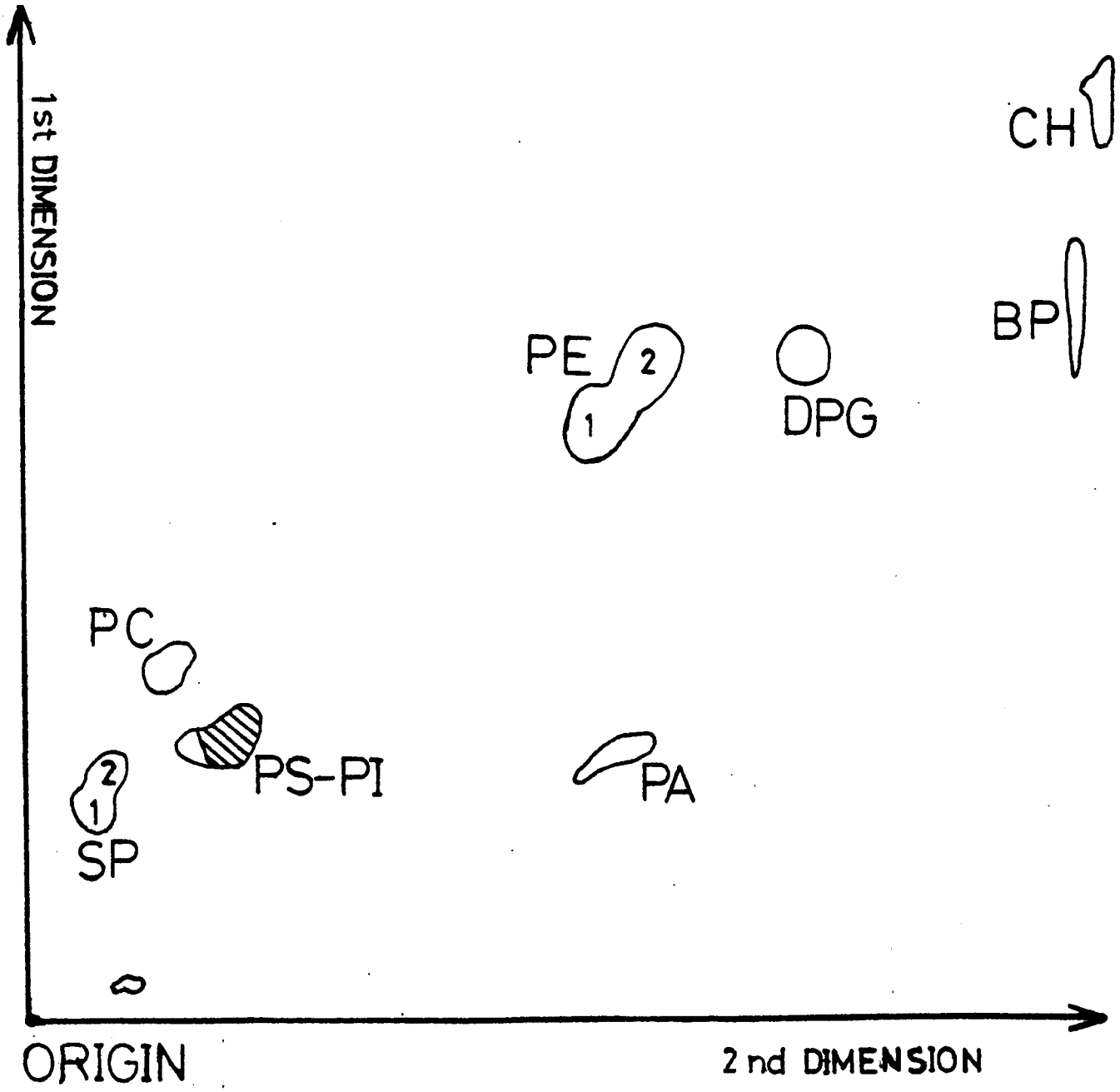

1st DIMENSION
CH
BP
PE
2
1
DPG
PC
PS-PI
PA
2
1
SP
ORIGIN
2 nd DIMENSION

FIGURE 8.

reagent (Table 4). Similarly, spots 9, 10 and 11 did not react with any of the specific spray reagents, except sometimes with the molybdenum spray reagent, (Table 4). These components were probably present at concentrations at or below the limits of sensitivity for chemical spray detection.

Some of the commercial standards and the microtubule-associated phospholipids migrated as double spots on thin-layer chromatograms (Figures 7 and 8). In all cases the appropriate specific spray reagent reacted with both parts of the chromatogram component. This suggested that there may have been heterogeneous populations of phospholipids of the same polar group, but different fatty acid composition. For this reason the R_f of both parts of the double spot were calculated to assist in identification. This effect was particularly evident with sphingomyelin (SP), phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE), although variation was observed between chromatograms run over different distances. In later chromatograms (particularly Figures 13 and 14), there were differences in relative mobilities due to separation over shorter distances although chromatogram patterns remained the same. In particular cardiolipin (DPG), in these circumstances, moved much closer to the solvent front in the second dimension. Chromatogram components 2 and 3 (Figure 7) were identified as SP components 1 and 2 from the chromatogram of phospholipid standards (Figure 8) due to their similar R_f values in both dimensions and their reaction with benzidine spray reagent.

Spot 4 contained mainly phosphatidyl serine (PS) as determined by R_f and a positive reaction with ninhydrin. However, only approximately 75% of this spot area (after visualisation with iodine vapour) reacted with ninhydrin which suggested that some other component was also present. Phosphatidyl inositol (PI) co-migrated with PS on the chromatogram of phospholipid standards (Figure 8) and only the head region of the spot reacted with ninhydrin. This suggested that the other component in spot 4 was PI.

Spots 5 and 6 were identified as PC by their R_f and the fact that they were the only chromatogram components to react with Dragendorff spray reagent. Spot 7 reacted with ninhydrin, which indicated the presence of a free amino group, and was similar in R_f to the PE standard. Spot 12 was identical in R_f to the cholesterol standard and reacted positively with the cholesterol spray reagent.

In summary then, the major phospholipids associated with one-cycle-purified microtubules were identified as PE, PC, SP and PS-PI. Four other unidentified phospholipids were also detectable by charring, but only in trace amounts. Cholesterol was also present in association with the microtubule pellet.

2.2 Comparison of microtubule-associated phospholipids with those of a crude brain homogenate.

The chromatogram pattern of microtubule-associated phospholipids (Figure 7) was similar to that of phospholipids extracted from a crude brain homogenate which had been

used as starting material for microtubule preparation (Figure 9). However, a few of the crude homogenate chromatogram components (spots A, B, C on Figure 9) were not present in chromatograms of microtubule-associated phospholipids. In addition, three of the crude homogenate components, which corresponded in R_f to spots 9, 10 and 11 of microtubule-associated phospholipids (Figure 7) were more easily detectable by charring than in the microtubule-associated phospholipid extract. This indicated that there may be differences in the relative amounts of individual phospholipids associated with the two sub-cellular fractions.

2.3 Analysis of phospholipids associated with highly-purified microtubules.

PC, PE, SP and PS-PI were all shown to persist in association with microtubules after one, two and three cycles of purification (Figures 10a, 10b and 10c). Cholesterol also persisted up to three cycles of microtubule purification, but the trace components (spots 1, 9, 10 and 11 in Figure 7) were not detectable beyond one cycle when extracted from this amount of protein (2.5mg).

Several attempts were made to analyse the levels of individual microtubule-associated phospholipids, which could be scraped off chromatograms, using acid digestion followed by inorganic phosphate assay.

Three different published methods were used (Parker and Peterson, 1965; Bartlett, 1959; Rosenthal and Han, 1969) and in each case the levels of phospholipid phosphate

FIGURE 9

Two-dimensional tlc of phospholipids from a crude brain homogenate.

A crude brain homogenate suspended in RB, the starting material for microtubule preparation, was freeze-dried. Phospholipids were extracted and separated by two-dimensional tlc. Shown are photographs of two chromatograms which contained approximately (a) 30 nmoles and (b) 150 nmoles of total phospholipid phosphate. These values represented the lowest and highest values obtained for total phospholipid phosphate associated with 2.5mg of one-cycle-purified microtubule proteins (see Figure 11). After visualisation by charring, it was possible to make a visual qualitative comparison of the relative amounts of components in these chromatograms with those shown in Figure 7. Abbreviations are the same as for Figure 7 except for A, B and C, which represent unidentified components not detected in microtubule preparations.

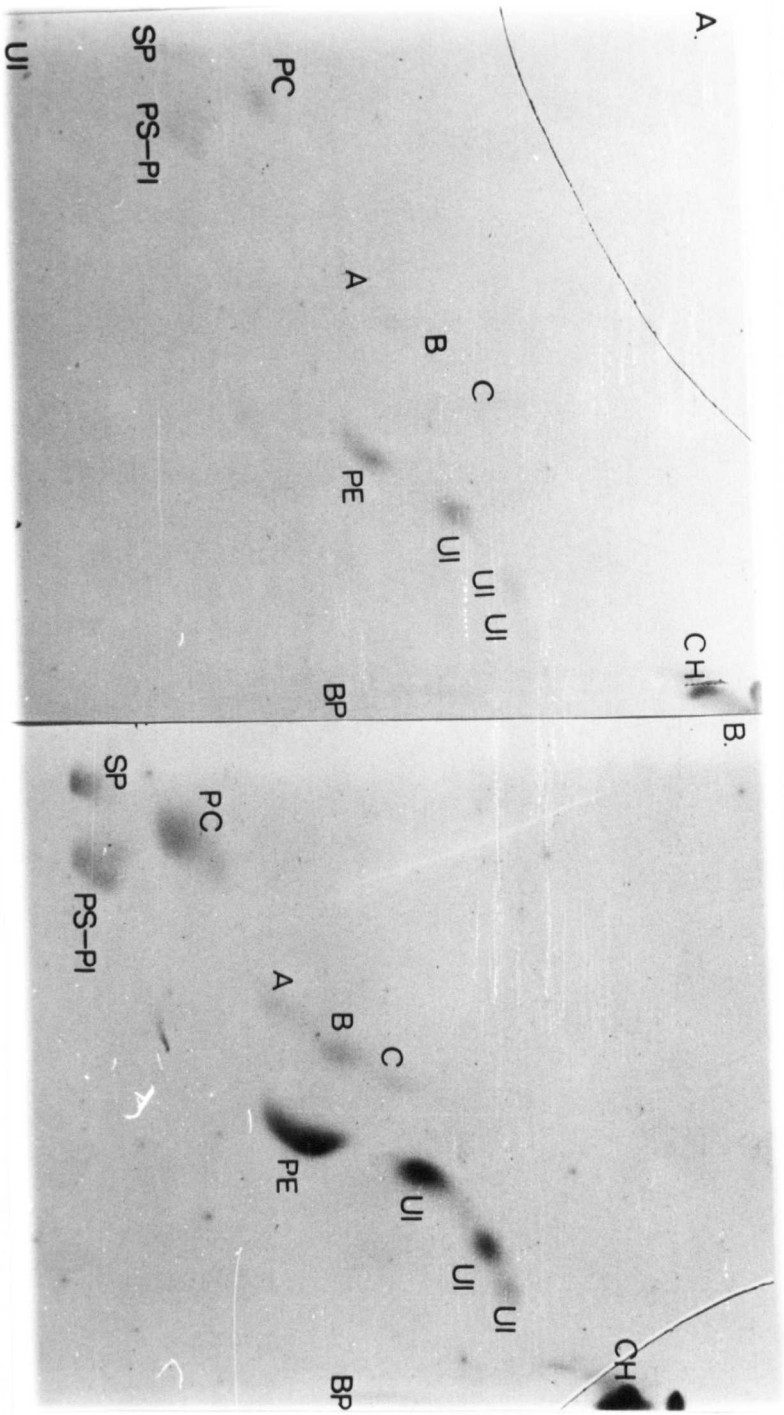

A.
CH
UI
UI
UI
C
B
A
PE
BP
PC
SP
PS–PI
UI
B.
CH
UI
UI
UI
C
B
A
PE
BP
PC
SP
PS–PI

FIGURE 9

FIGURE 10

Two-dimensional tlc of phospholipds associated with highly-purified microtubules.

Microtubules were pelleted by centrifugation after (a) one, (b) two or (c) three cycles of temperature-dependent recyclization. Phospholipids were extracted from a fixed amount (2.5mg) of microtubule protein at each stage of purification, in quadruplicate, and separated in parallel by two-dimensional tlc. Visualisation was by charring and chromatogram components were identified as indicated.

Abbreviations are as shown in Figure 7.

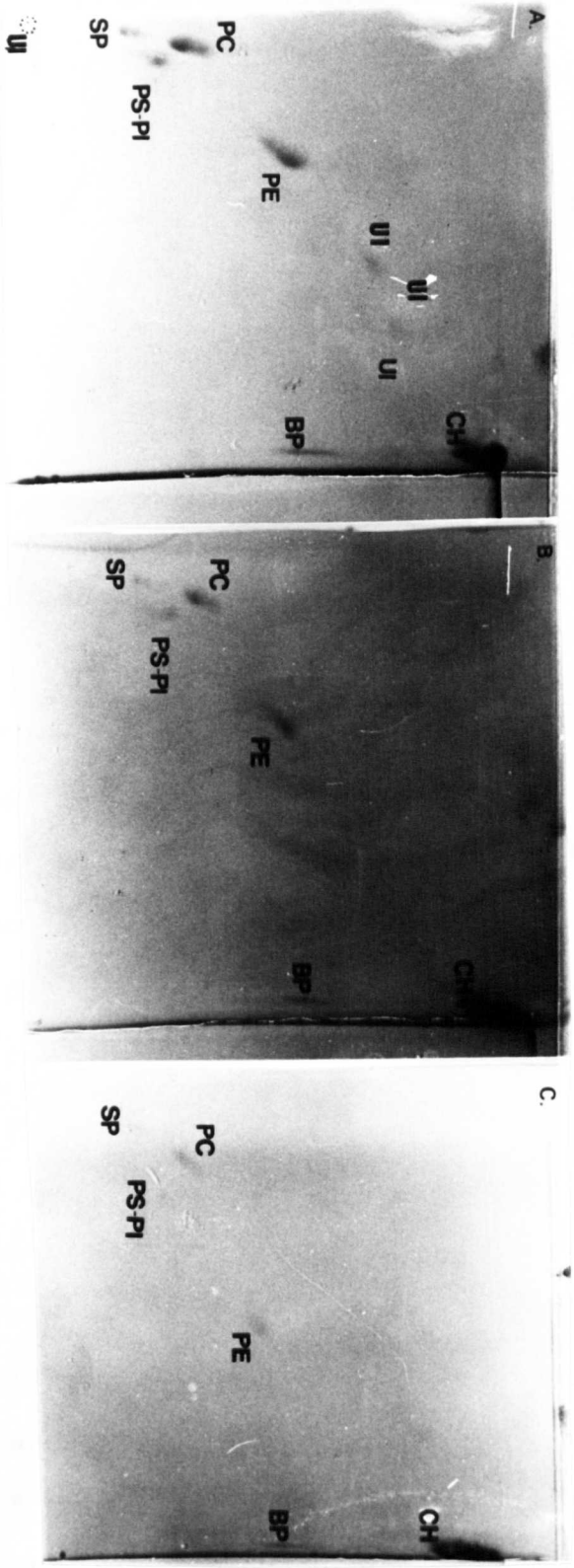

A
SP
PC
PS-PI
PE
UI
UI
UI
UI
BP
CH
B
SP
PC
PS-PI
PE
BP
CH
C
SP
PC
PS-PI
PE
BP
CH

FIGURE 10

present in individual chromatogram components were too low to be detectable by phosphate assay (results not shown). When standard phospholipids, after two dimensional tlc, were analysed by the same methods it was calculated that at least a ten-fold increase in the amounts of phospholipid chromatographed would be necessary for individual microtubule-associated phospholipids to be detectable by phosphate assay. Such a scale-up in amounts of material for analysis was not practical due to limitations on the number of pig brains available for microtubule extraction over a given period of time and so some other qualitative approach had to be adopted. It was decided to use a combination of densitometry and analysis of total phospholipid phosphate.

The peak areas from densitometry of all the major chromatogram components detectable by charring at one, two and three cycles of microtubule purification decreased to a similar extent relative to their levels at one cycle (Table 5). The percentage decrease of each component was greater than that of total phospholipid phosphate between one and two cycles of microtubule purification, but was similar after three cycles.

This only applies to the few phospholipids (PC, PE, PS-PI, SP) which were readily detectable by charring up to three cycles of microtubule purification. It is conceivable that the relative changes in other components not easily detected by charring (e.g., spots 1, 9, 10, 11 on Figure 7) may be different.

Further analysis of total phospholipid phosphate showed that, after an initial large decrease in the levels

TABLE 5

Densitometric analysis of the major phospholipids associated with highly-purified microtubules.

PHOSPHOLIPID	RELATIVE PEAK AREA AT VARIOUS CYCLES OF MICROTUBULE PURIFICATION		
	ONE	TWO	THREE
PE	100 ± 11.7	30.4 ± 2.8	12.0 ± 0.6
PC	100 ± 11.0	38.1 ± 3.9	21.1 ± 0.9
SP	100 ± 5.1	38.8 ± 6.5	14.6 ± 1.4
PS-PI	100 ± 7.4	27.6 ± 1.9	12.9 ± 1.7
PLP	100 ± 22.1	53.4 ± 2.5	16.5 ± 4.1

Chromatogram components from the experiment described in Figure 10 were analysed by densitometry. Densitometric peak areas were cut out, from constant weight paper, and weighed. Relative peak areas at two and three cycles of microtubule purification are expressed for each component as a percentage of their value at one cycle ± S.E.M. of four determinations (from four separate chromatograms at each stage of purification). Total phospholipid phosphate (PLP) was also determined at each stage of purification.

Other abbreviations are as shown in Figure 7.

of phospholipid phosphate relative to protein between one and three cycles of microtubule purification, there was an enrichment at five cycles to a level of 23.8 nmol per milligram total protein (Figure 11). Presumably this reflected comparable increases in the levels of the major individual microtubule-associated phospholipids described above. However, it was not possible to show whether this was the case due to the lack of materials required to carry this analysis further. Therefore, the possibility that different changes may have occurred in the relative levels of some microtubule-associated phospholipids on purification between three and five cycles cannot be ruled out.

The phospholipid phosphate assay used in this thesis relies on the release of inorganic phosphate after incubation with perchloric acid (Bartlett, 1959). It was important to establish that the readings obtained for phospholipid phosphate were not contaminated by traces of GTP associated with the microtubule proteins. Assays performed on 100 and 200µl of RB which contained 0.5mM GTP produced zero readings. Other controls which included samples without acid digestion produced similar results.

2.4 Analysis of phospholipids associated with phosphocellulose-purified tubulin and accessory proteins

The three-cycle-purified microtubule proteins used in these experiments contained 70 - 75% tubulin as estimated by densitometry of microtubule proteins separated by SDS-PAGE. If it is assumed that all of the

FIGURE 11

Analysis of total phospholipid phosphate associated with one-, three- and five-cycle purified microtubules.

Microtubules were pelleted by centrifugation after one, three or five cycles of temperature-dependent recyclization. Phospholipids were extracted from 5 - 10mg of microtubule protein and assayed for total phospholipid phosphate. Results are expressed as mean ± S.E.M. of five or more determinations which were pooled from two separate microtubule preparations at one and three cycles, or one preparation at five cycles. Student's 't' test; one cycle vs. three cycles, $p<0.001$; one cycle vs. five cycles, $p<0.01$; three cycles vs. five cycles, $p<0.0005$.

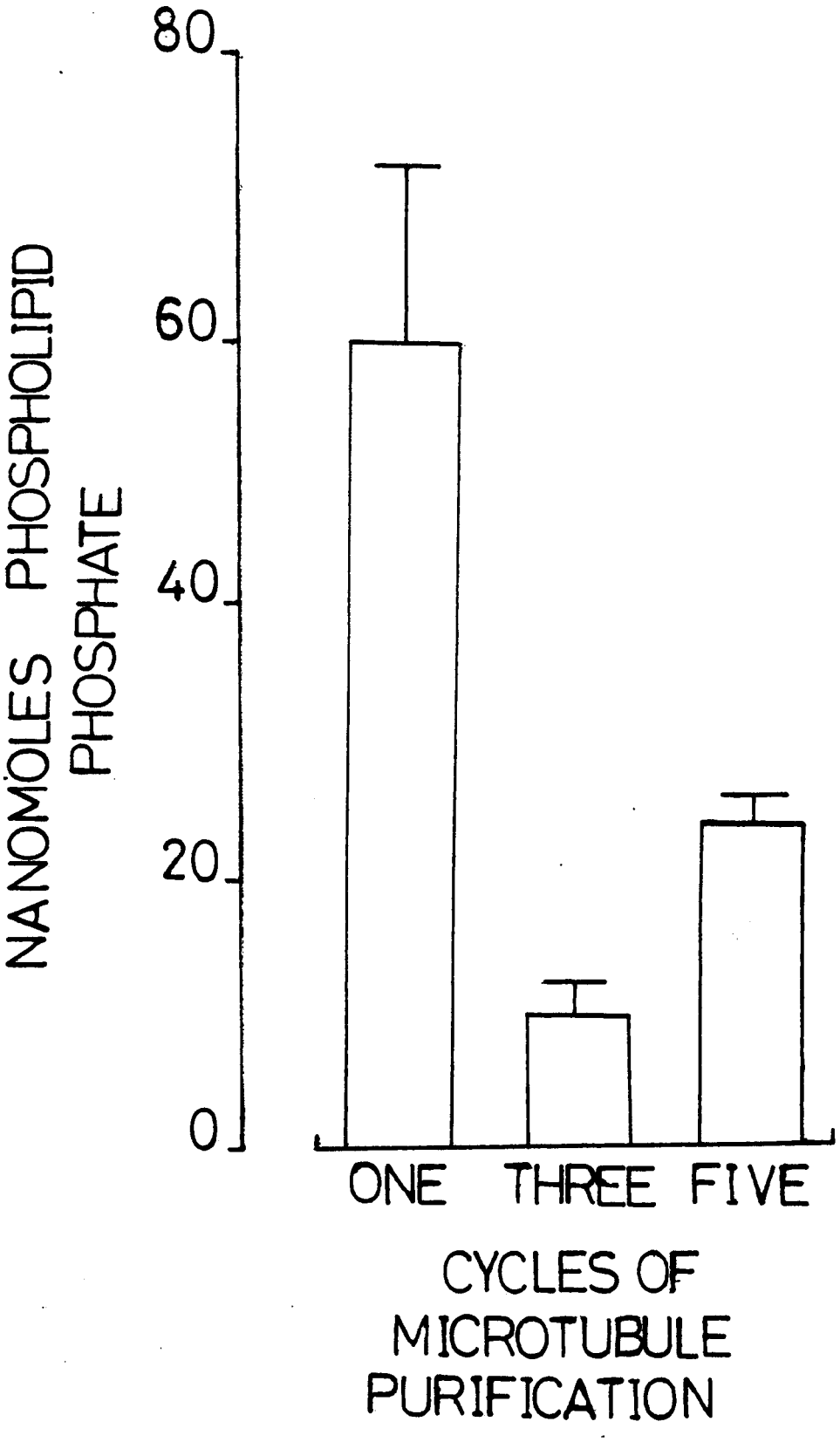

80
60
40
20
0
NANOMOLES PHOSPHOLIPID PHOSPHATE
ONE
THREE
FIVE
CYCLES OF MICROTUBULE PURIFICATION

FIGURE 11

phospholipids are associated with tubulin, a level of 1.5 moles phospholipid phosphate per mole tubulin dimer can be calculated from the value obtained for three-cycle-purified microtubule-associated phospholipids (Figure 11). This value would then be doubled at five cycles of microtubule purification.

However, as Kirazov and Lagnado (1975) demonstrated the presence of some phospholipid phosphate in a high molecular weight accessory protein-enriched fraction of rat brain microtubule proteins separated on sepharose 6B, it was possible that some phospholipids were associated with accessory proteins in the microtubule preparations used in the present study. To determine whether this was the case, it was decided to carry out further qualitative and quantitative analyses on microtubule proteins fractionated by phosphocellulose chromatography.

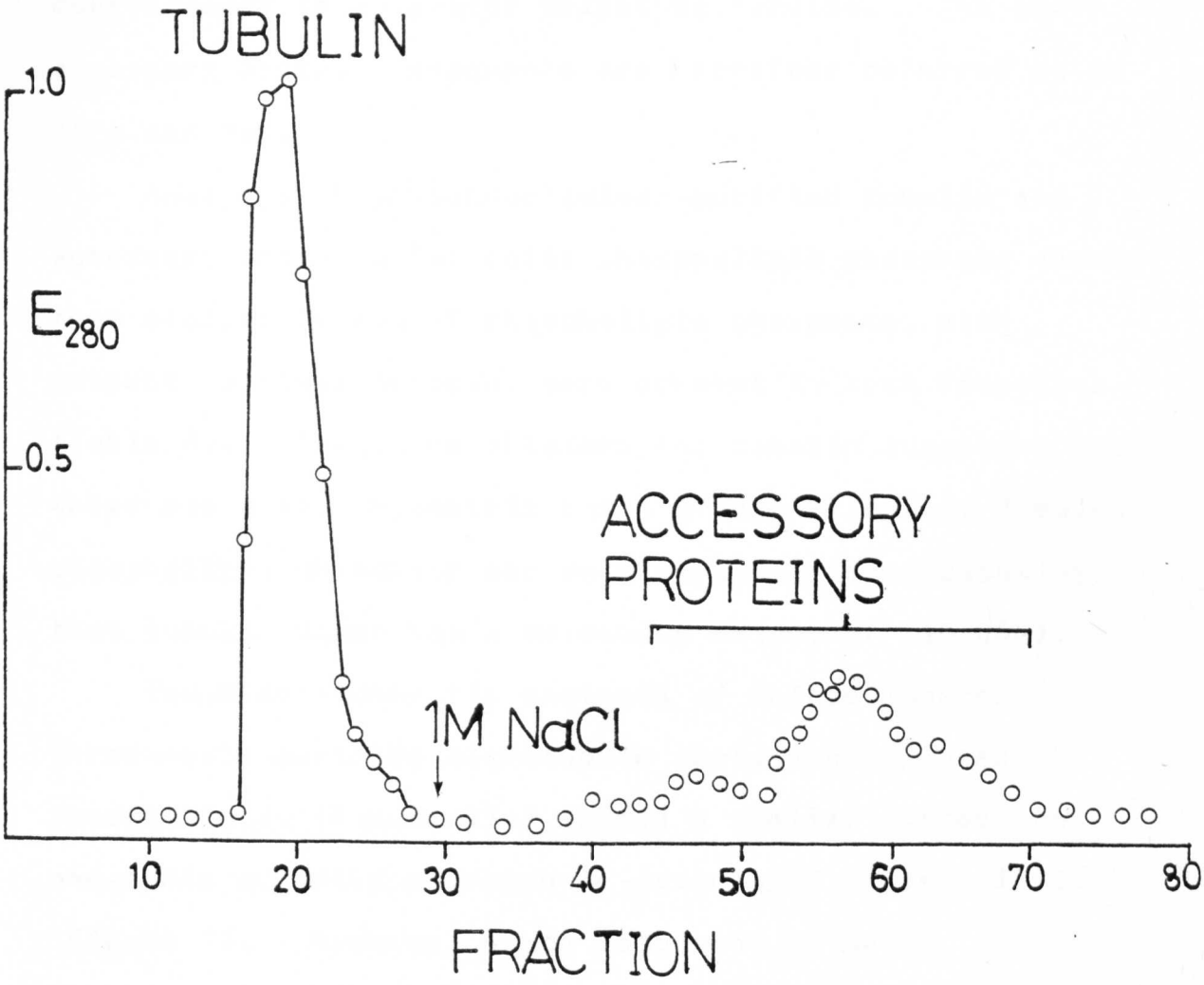
The phosphocellulose col umn was prepared and run as described in Methods, Section 4. Tubulin, as expected, was eluted in the void volume whereas the accessory proteins (which bound to the phosphocellulose resin) were eluted with 1M NaCl (Figure 12). Analysis of unfractionated microtubule proteins, phosphocellulose-purified tubulin and accessory proteins showed that the tubulin fraction was homogeneous and that the accessory protein fraction contained low and high molecular weight proteins which corresponded in molecular weight to MAPs (Murphy and Borisy, 1975; Murphy, Vallee and Borisy, 1977; Sloboda, Dentler and Rosenbaum, 1976) and tau factor (Witman et al., 1975; Cleveland et al., 1977) and a component which

FIGURE 12.

Separation of tubulin from accessory proteins by phosphocellulose chromatography.

Microtubules were pelleted by centrifugation after three cycles of temperature-dependent recyclization. The microtubule pellet was then resuspended in ice-cold column buffer (20mM PIPES, 1mM EGTA, 0.5mM $MgSO_4$ at pH 6.8) to a concentration of 5 - 10 $mg.ml^{-1}$, incubated for 30 minutes on ice and then clarified by centrifugation. The protein suspension was then applied to a phosphocellulose column, equilibrated at 4°C with column buffer and chromatographed at a flow rate of 15 - 30 $ml.hour^{-1}$. Fractions of 2 ml were collected and their absorbance measured at 280nm. Shown is a typical column profile (one from three experiments) in which the first peak contained tubulin and the second peak, which was eluted with 1M NaCl, contained accessory proteins. Recovery of protein was approximately 70%.

Shown below are photographs of polyacrylamide gels containing (a) 40µg of unfractionated microtubule proteins (b) 80µg of phosphocellulose-purified tubulin and (c) about 10µg of accessory proteins.


TUBULIN
1.0
E_{280}
0.5
ACCESSORY
PROTEINS
1M NaCl
10
20
30
40
50
60
70
80
FRACTION

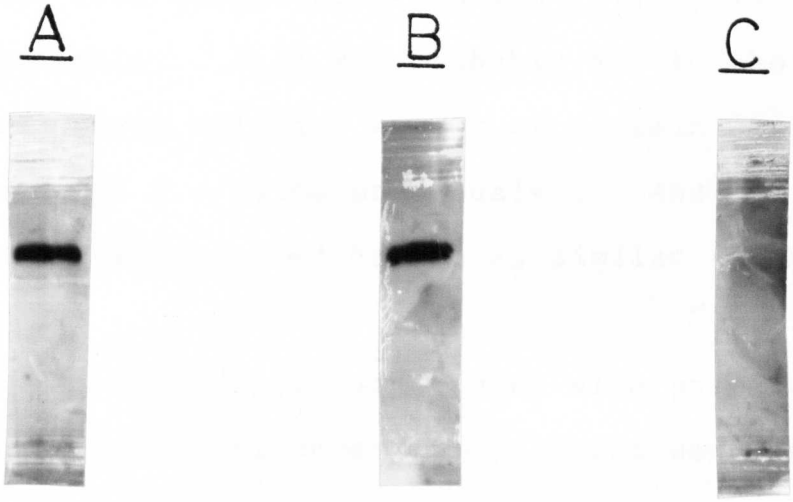

A
B
C

FIGURE 12.

corresponded in molecular weight to tubulin. The above accessory protein components are hereafter referred to as MAPs and tau.

Analysis of phosphocellulose-purified tubulin and accessory proteins for total phospholipid phosphate showed that similar levels of phospholipid phosphate, with respect to total protein, were present in both fractions (Table 6). The value obtained for tubulin suggested that there was a stoichiometric ratio of approximately 2 moles phospholipid phosphate per mole tubulin dimer (assuming that tubulin dimer has a molecular weight of 110,000).

Two-dimensional tlc analysis of unfractionated three-cycle-purified microtubule protein-associated phospholipids (Figure 13a) showed a similar pattern to one-cycle purified microtubule-associated phospholipids (Figure 7). However, trace component 1 (N^+ in Figure 7), which had not previously been detected at three cycles of microtubule purification (Figure 10c), were now detectable. This was probably due to phospholipid extraction from a larger amount of protein (5 - 6mg compared with 2 - 2.5mg previously). Another component not previously detected had an R_f similar to the DPG standard.

When phospholipids associated with phosphocellulose-purified tubulin and accessory proteins were analysed by tlc, differences were observed in the number and type of phospholipids present in each fraction. Comparison of chromatogram components from unfractionated microtubule protein (Figure 13a) with those from tubulin (Figure 13b)

TABLE 6

Analysis of total phospholipid phosphate associated with phosphocellulose-purified tubulin, accessory proteins and unfractionated microtubule protein.

FRACTION	NANOMOLES TOTAL PHOSPHOLIPID PHOSPHATE PER MILLIGRAM PROTEIN			
Tubulin	20.28	±	5.64	(n = 7)
Accessory Protein	21.72	±	2.94	(n = 6)
Unfractionated microtubule protein	17.99	±	2.44	(n = 3)

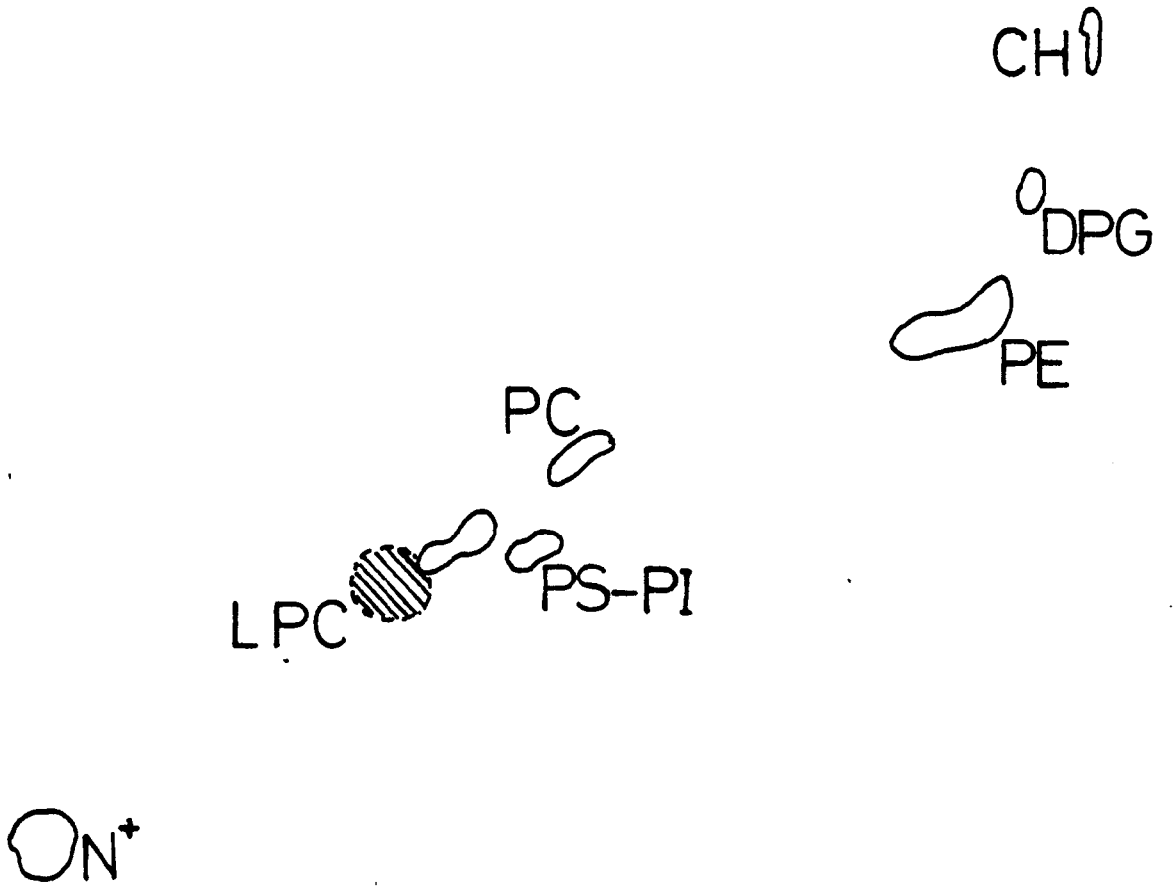
Three-cycle purified microtubule protein was separated by phosphocellulose chromatography into two components which contained either tubulin or accessory proteins. Phospholipids were extracted from both fractions and from unfractioned microtubule protein, using the Bligh and Dyer (1959) procedure, and then assayed for total phospholipid phosphate. The results are expressed as a mean ± S.E.M. of 'n' determinations, as indicated, pooled from separate assays on protein from a single microtubule preparation.

FIGURE 13.

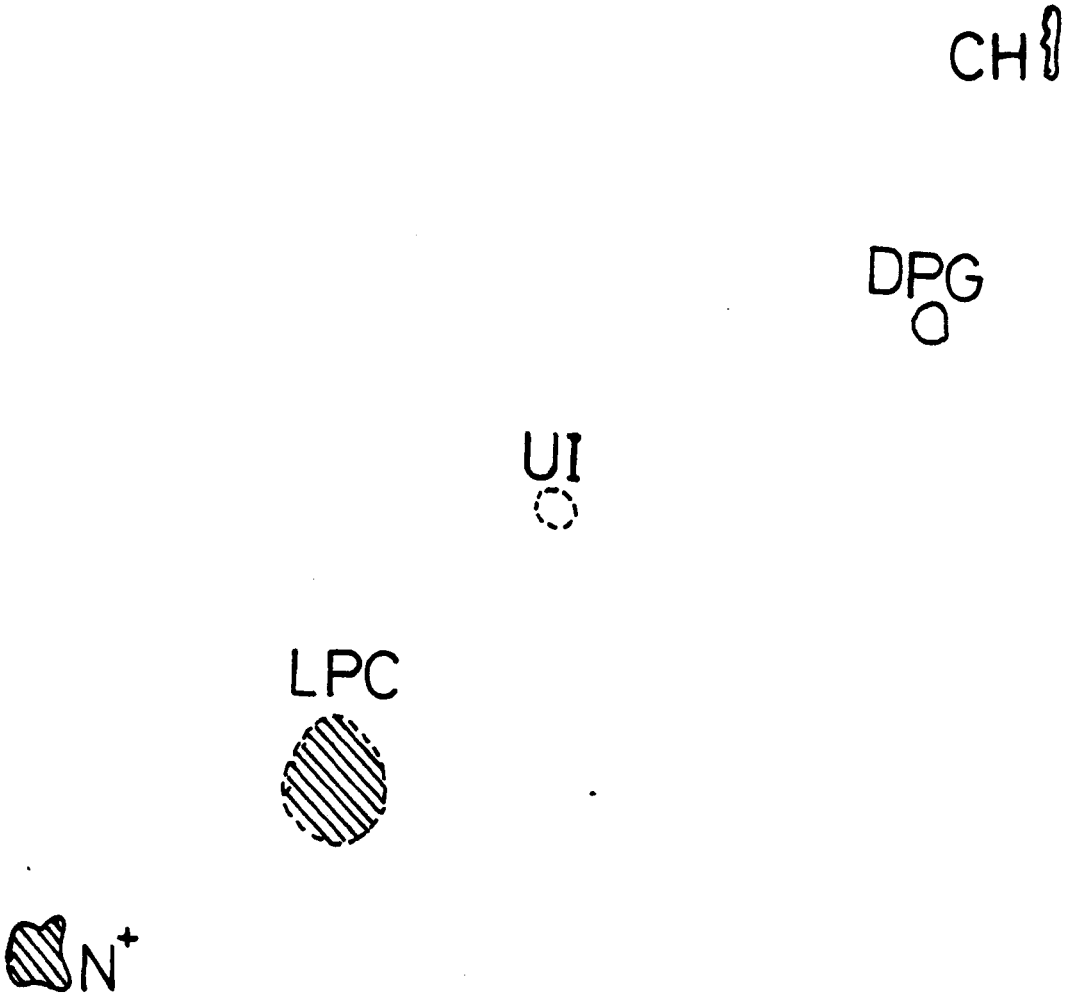
Two-dimensional tlc of phospholipids associated with tubulin, accessory proteins and unfractionated three-cycle-purified microtubule proteins.

Three-cycle-purified microtubule proteins were fractionated by phosphocellulose chromatography into two components which contained either tubulin (B) or accessory proteins (C). Phospholipids were extracted from 5 - 6mg from both fractions and a sample of unfractionated microtubule proteins (A) and then separated (about 15cm each dimension) by two-dimensional tlc. Shown are schematic representations of typical plates (one of three experiments) after visualisation by iodine vapour, followed by ninhydrin reagent and finally charring. The plates were then observed under ultraviolet light. The components were identified by comparison with standards as indicated. Solid lines encircle components which were detectable by charring. Dotted lines encircle components visualised by iodine, but not detectable by charring. Shaded components were fluorescent under ultraviolet light. N^+ represents an unidentified component which gave a positive reaction with ninhydrin reagent. LPC is an abbreviation for lysophosphatidyl choline and all other abbreviations are as described in Figure 7.

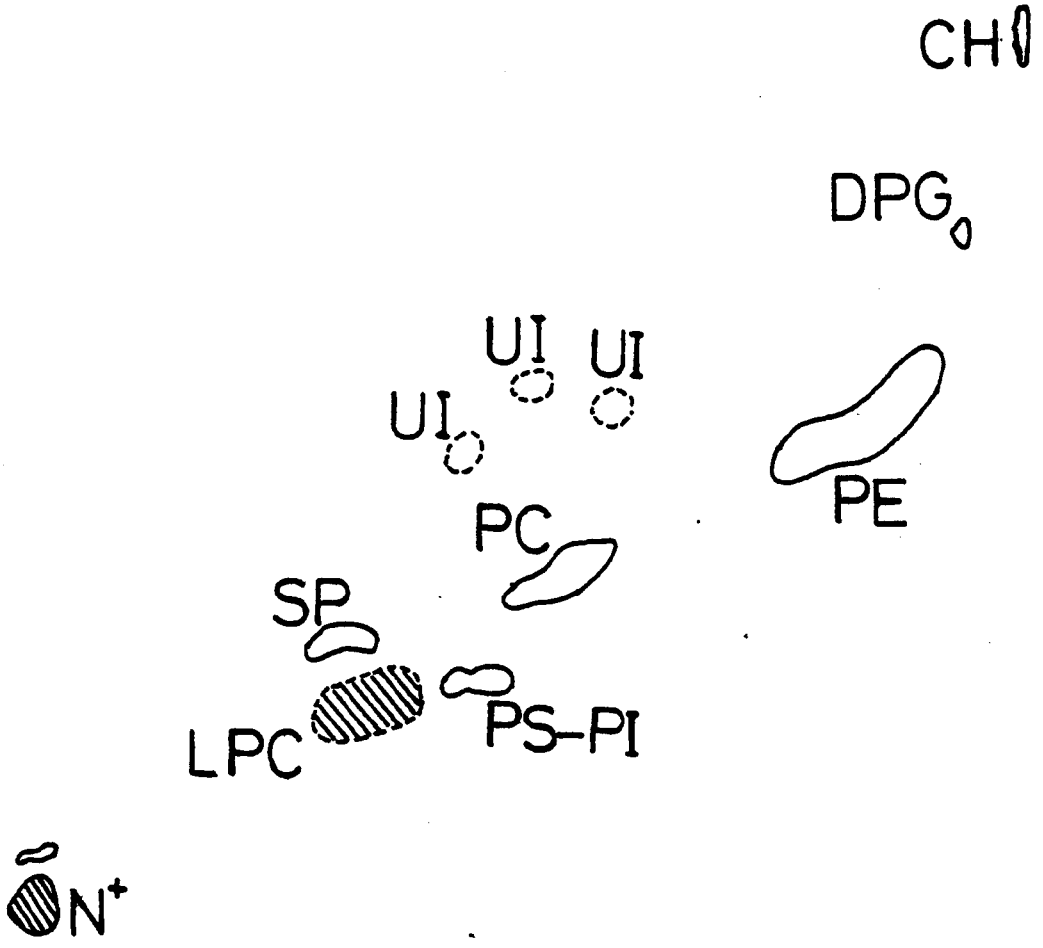
A.



B.



C.



and accessory proteins (Figure 13c) suggested that most of the phospholipids detectable by charring (i.e., PC, PE, SP, PS-PI) were present in accessory protein fractions, but not in the tubulin fractions. Some of the trace components (UI), which were only visible by iodine staining, appeared to be enriched in accessory proteins compared to whole microtubule extracts (compare Figure 13a with Figure 13c). One of these components (UI) appeared in the tubulin fraction (Figure 13b). The origin spot, present in all chromatograms, reacted with ninhydrin but did not co-migrate with a lysophosphatidyl ethanolamine standard. One other component, which had not been previously detected in whole microtubule extracts (Figure 7) was detectable by iodine and sometimes after excitation under ultra violet light (Figures 13a, 13b, 13c). This component, which was present in all three protein fractions, appeared to be most abundant in the tubulin fraction as determined by the larger spot size and intensity of iodine staining (two experiments) and was identified as lysophosphatidyl choline (LPC).

Cholesterol still appeared to be present in all chromatograms and one other spot which was particularly abundant in tubulin fractions, as shown by chromatograms run with low concentrations of total phospholipid phosphate (Figure 14), was tentatively identified by mobility and spot shape as DPG. The development of this spot was found to be enhanced by charring for two hours instead of one hour as done previously.

FIGURE 14

Tentative identification of cardiolipin as a major tubulin-associated phospholipid.

Three-cycle-purified microtubule proteins were separated by phosphocellulose chromatography into two components which contained either tubulin or accessory proteins. Phospholipids were then extracted from the tubulin fraction by the Bligh and Dyer procedure (1959), assayed for total phospholipid phosphate, and separated by two dimensional tlc. Visualisation was by charring.

Shown is a photograph of a typical chromatogram containing 10 nmoles of total phospholipid phosphate.

Only cardiolipin (DPG) and a breakdown product (BP) were detected at this concentration.

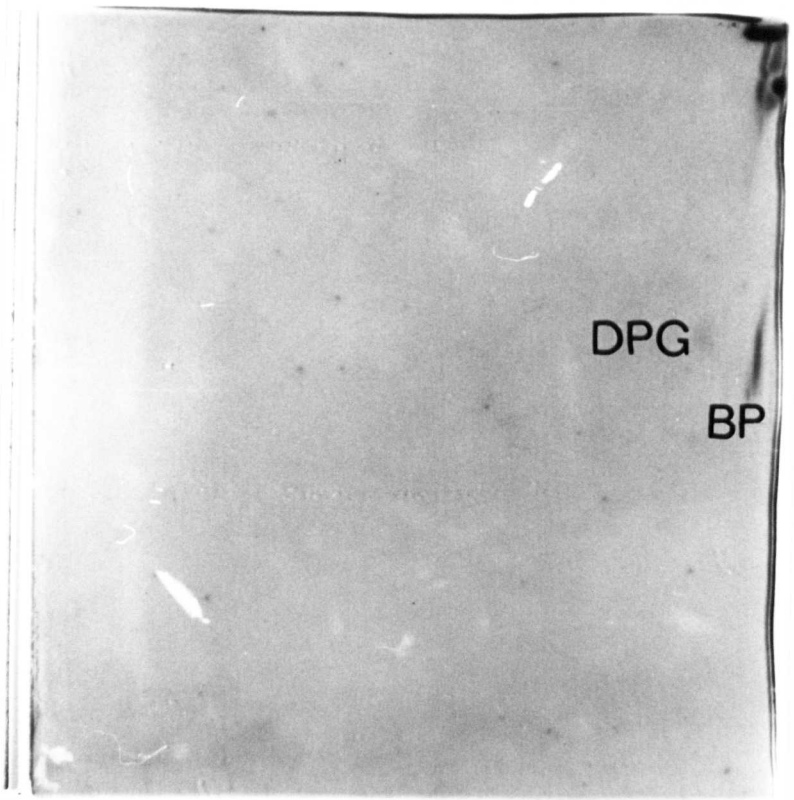


FIGURE 14

It was necessary to show that the observed distribution of phospholipids was not an artifact of interactions between them and the phosphocellulose resin. For example, the polar head-groups of some phospholipids may interact with the charged groups on the resin, causing the retention of those lipids on the column. This could modify the actual distribution of phospholipids eluted from the column if these interactions were strong enough to overcome the protein-phospholipid associations. For this reason a sonicated aqueous suspension of all the commercial phospholipid standards was separated on a phosphocellulose column, under identical conditions to microtubule protein separation, and the fractions which had previously contained tubulin and accessory proteins were again pooled and analysed for phospholipid content by two-dimensional tlc. It was clearly shown that most of the phospholipids which had been applied to the column appeared in the fractions which would normally contain tubulin (Figure 15). Only traces of lipid were eluted in the equivalent accessory protein fractions. This suggested that the phospholipids would normally have behaved differently on the column if they had not been in some way associated with microtubule proteins.

FIGURE 15

Phosphocellulose chromatography of a sonicated suspension of phospholipid standards.

A mixture of phospholipid standards which contained 50µg each of PC, PE, SP, PS, PI, PA and DPG were evaporated to dryness *in vacuo*. They were then resuspended by sonication into 2ml column buffer at 4°C and chromatographed under identical conditions to microtubule proteins in Figure 16. The fractions which corresponded to tubulin and accessory protein on the elution profile on Figure 16 were then collected, pooled separately, and a phospholipid extraction performed. Phospholipids were then separated by two-dimensional tlc, on plates divided into four small chromatograms, for 7cm each dimension, and visualised by charring. Shown are photographs of (a) unfractionated phospholipid suspension (b) phospholipids which eluted as for tubulin and (c) phospholipids which eluted as for accessory proteins. The amount of unfractionated phospholipids separated corresponded to approximately 2/5ths of total phospholipids chromatographed on the column.

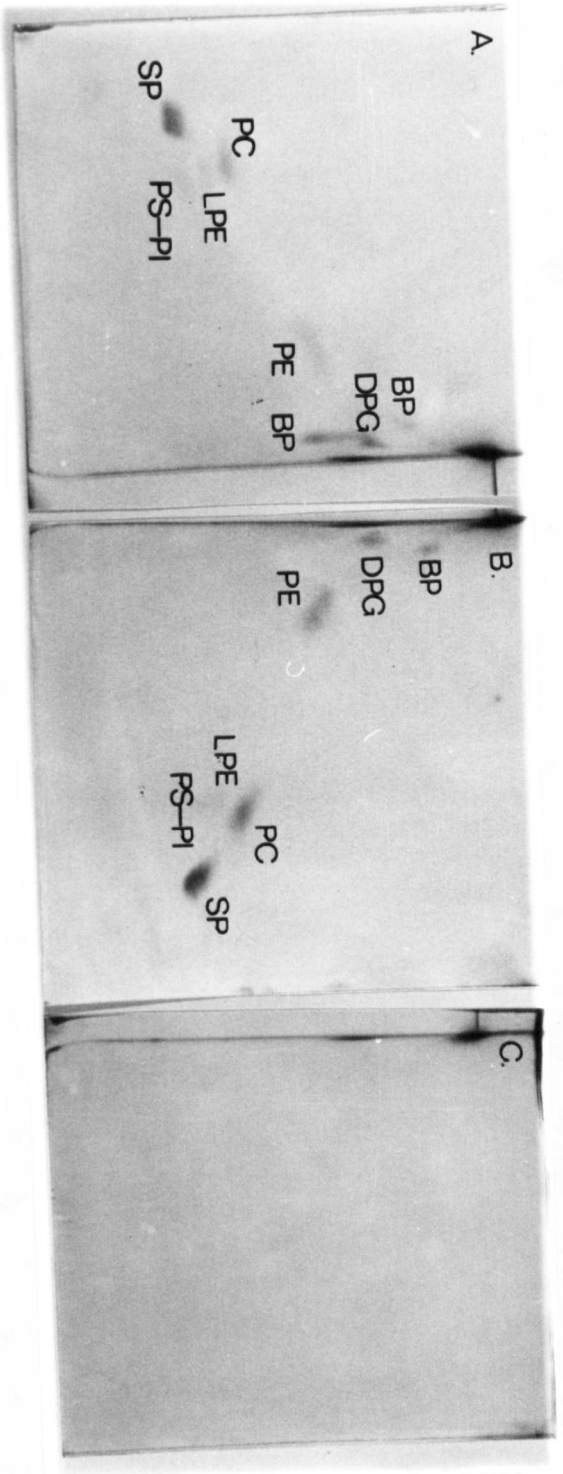


FIGURE 15.

3. DO PHOSPHOLIPIDS INFLUENCE MICROTUBULE ASSEMBLY?

The results in Section 2 show that there is a persistent association of a number of phospholipids even with highly-purified microtubule proteins. In order to determine whether this reflected a regulatory role of these phospholipids in microtubule assembly, experiments were carried out to study the effects of exogenous phospholipids and phospholipases on microtubule assembly *in vitro*.

3.1 The effects of phospholipases and phospholipase inhibitors on microtubule assembly.

In a typical experiment, microtubule proteins at a concentration of 2 - 3mg.ml^{-1} were pre-incubated with phospholipase A_2 (*V.russelli*) or phospholipase C (*C.welchii*) at 37^{o}C for 15 minutes in the absence of GTP. GTP was finally added to initiate microtubule assembly. It was found that phospholipases had no effect on microtubule assembly unless a pre-incubation was performed. In some experiments the presence of 0.5mM Ca^{2+} in the pre-incubation medium, which consisted of RB lacking EGTA (pre-incubation was terminated, before the initiation of microtubule assembly, by the addition of 1mM EGTA to chelate free Ca^{2+}), there was an enhancement of the subsequent inhibition of microtubule assembly. However, a pre-incubation with Ca^{2+} alone also resulted in a level of inhibition of microtubule assembly which accounted for the difference between Ca^{2+}-free and Ca^{2+}-incubated preparations containing phospholipases and microtubule proteins (results not shown). For this reason, Ca^{2+}-free buffer was used for all of the

experiments described in this Section as it was suspected that the Ca^{2+} effect may be caused by some other pathway such as a Ca^{2+}-activated protease (Sandoval and Weber, 1978).

Both phospholipase A_2 and phospholipase C inhibited microtubule assembly (Table 7). Their inhibitory effects were not reversed in the presence of a soya bean trypsin inhibitor, but the effects of phospholipase A_2 were partially reversible in the presence of 20μM Dimethyl-D,L-2,3-distearoyloxypropyl-2' hydroxyethyl ammonium acetate (a PC analogue which competitively inhibits phospholipase A_2 activity {Rosenthal and Geyer, 1960}). This suggested that the commercial phospholipase was acting on PC present in the microtubule extracts. As expected, there was no reversal of the effects of phospholipase C by the PC analogue as this enzyme acts on a different substrate (see Appendix 2).

Analysis of microtubule-associated phospholipids showed that a number of phospholipids were hydrolysed after incubation with phospholipase C (Figure 16). There was an increase in the intensity of iodine staining in the top right-hand corner of the chromatogram of phospholipase C-treated preparations (Figure 16b) which probably reflected the accumulation of 1,2-diglyceride. However, it was interesting to note the appearance of a spot on the same chromatogram which corresponded in R_f to phosphatidic acid (PA). This result agreed with the findings of Daleo et al., (1974), who discovered a diglyceride kinase activity in their microtubule preparations. The production of PA

TABLE 7

The effects of phospholipases, phospholipase inhibitors and a trypsin inhibitor on microtubule assembly.

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2 - 3mg.ml^{-1}, were pre-incubated at 37°C for 15 minutes in the presence of 0.5 units.ml^{-1} of phospholipase C or 0.22 units.ml^{-1} of phospholipase A_2. Microtubule assembly was then initiated by the addition of 0.5mM GTP and monitored as a change in optical density (350nm) at 37°C. In some experiments, where indicated, a PC analogue (Dimethyl-DL-2,3-distearoyloxypropyl-2' hydroxyethyl ammonium acetate) was added, before the pre-incubation, at a concentration of 20µM. In other samples a soya bean trypsin inhibitor or another phospholipase inhibitor p-bromophenacyl bromide (pBPAB) were added at a concentration of 10µg.ml^{-1}. The initial rate of microtubule assembly (R) and the extent of microtubule assembly (ΔOD) were calculated from each spectrophotometer trace. Results are expressed as percentage of controls ± S.D. of 'n' experiments, as indicated. The statistical significance of the data was determined using paired 't' tests. The significance values (p) shown are from comparison with controls, or where indicated by an asterisk, from comparison with samples incubated with phospholipase.

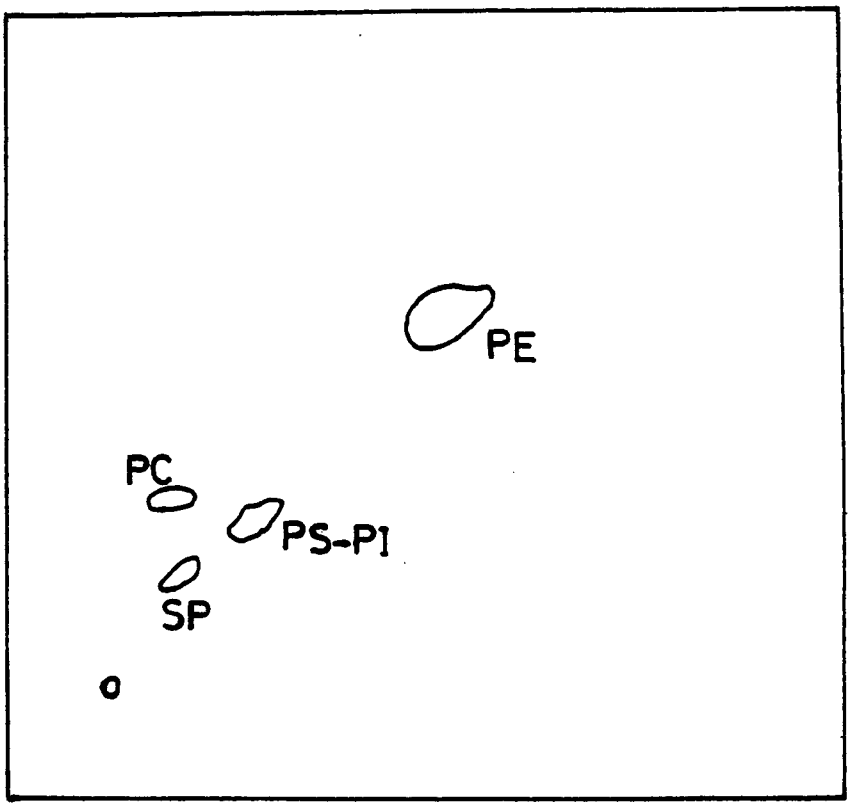
TABLE 7

ADDITIONS	ΔOD	% CONTROL ± S.D. p	R	p
Phospholipase A_2	79.3 ± 8.4 (n = 9)	<0.001	74.1 ± 12.6 (n = 8)	<0.001
Phospholipase A_2 + PC analogue	88.7 ± 8.1 (n = 6)	<0.02 <0.01*	97.0 ± 14.0 (n = 5)	NS 0.1>p>0.05*
Phospholipase A_2 + Trypsin inhibitor	71.2 ± 9.9 (n = 4)	NS*	65.8 ± 14.2 (n = 3)	NS*
Phospholipase A_2 + pBPAB	7.6 ± 1.0 (n = 2)		-	
Phospholipase C	82.0 ± 5.4 (n = 5)	<0.01	82.9 ± 5.8 (n = 4)	<0.02
Phospholipase C + PC analogue	85.6 ± 8.8 (n = 2)	NS*	-	
Phospholipase C + Trypsin Inhibitor	85.5 ± 1.7 (n = 2)	NS*	-	

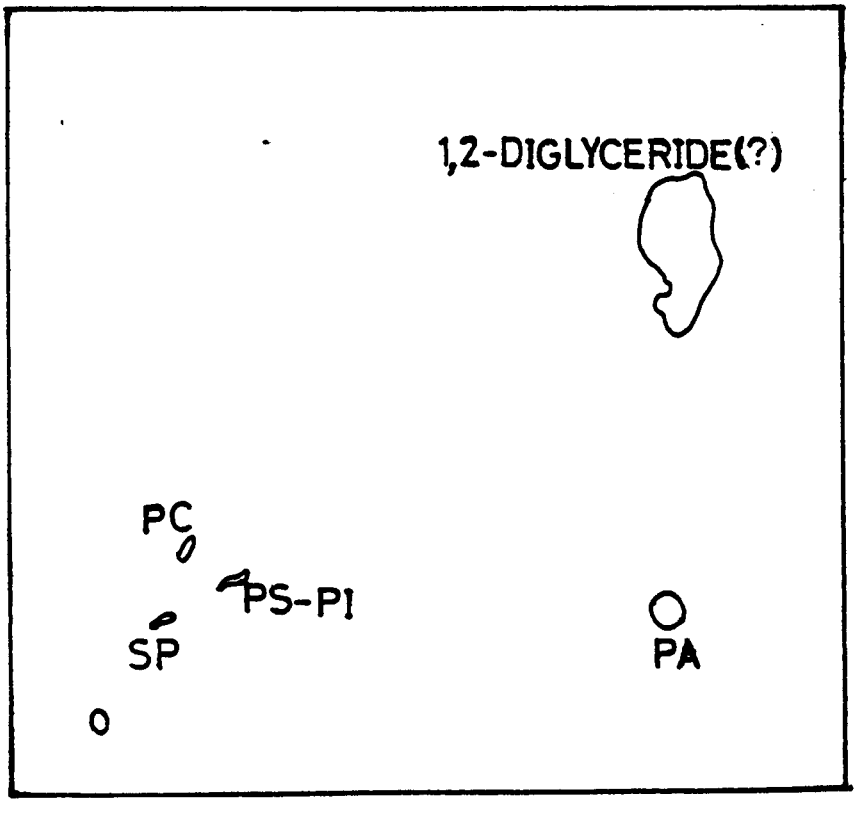
FIGURE 16

Two-dimensional tlc of the effects of phospholipase C on microtubule-associated phospholipids.

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 3mg.ml^{-1}, were pre-incubated at 37^{o}C for 15 minutes in the presence of 0.5 units ml^{-1} of phospholipase C. Microtubule assembly was initiated by the addition of 0.5mM GTP and allowed to proceed for 20 minutes. Microtubules were then pelleted by centrifugation at 20,000g for 45 minutes at 37^{o}C after which the resultant pellets were freeze-dried. Phospholipids were then extracted, analysed by two-dimensional tlc and visualised by iodine. Shown is a schematic representation of typical plates which contain (a) untreated microtubule-associated phospholipids and (b) phospholipase C-treated microtubule-associated phospholipids (one from two experiments). The major phospholipids PC, PS, PI, PE and PA are indicated.



A.



B.

FIGURE 16.

after incubation with phospholipase C may reflect the presence of a diglyceride kinase activity in my microtubule preparations.

One other phospholipase inhibitor, p-bromophenacyl bromide, was included in some experiments (Table 7). This drug is believed to inhibit phospholipase A_2 by binding at, or near to, a histidine residue close to the active site (Volwerk, Pieterson and De Haas, 1974). In the experiments where this drug was used (Table 7) microtubule assembly was almost completely inhibited even in the absence of phospholipase A_2. There is some evidence in the literature to suggest that a histidine residue may be involved in the regulation of microtubule assembly (Sakai, 1980). It may be that the p-bromophenacyl bromide interfered with such a histidine residue in my experiments. For this reason it is difficult to interpret this result as an effect on modification of microtubule-associated phospholipids and further experiments were not carried out.

3.2 The effects of phospholipases on microtubule assembly in the presence of sodium fluoride.

GTP was shown to be essential for the initiation of microtubule assembly under normal buffer conditions *in vitro* (Figure 1a). One possible way that the commercial enzyme preparations could have inhibited microtubule assembly was due to the presence of a contaminating GTPase activity. In fact phospholipase C, which acts on the phosphate ester moiety of phospholipids (see Appendix 2), would be the most likely phospholipase to remove the terminal phosphate from

GTP. It has been shown that the inclusion of 10mM NaF in the buffer medium inhibits GTPase activity, in phospholipase C preparations, to a level which does not interfere with microtubule assembly (Daleo et al., 1977). Based on these authors' findings, 10mM NaF was included in the experiments described in Table 8, to inhibit any contaminating GTPase activity which may have been present in the commercial phospholipase preparations.

The data in Table 8 show that the extent of microtubule assembly was inhibited to a similar degree as in experiments where NaF was absent (Table 7). The rate of microtubule assembly in the case of phospholipase A_2 was also affected to a similar extent whether NaF was present or not. In the case of phospholipase C, however, the inhibition of the rate of microtubule assembly was slightly greater in the presence of NaF although this may have been due to the fact that a new batch of enzyme was used in these experiments. The results suggested that a contaminating GTPase activity was not responsible for the inhibition of microtubule assembly by phospholipases A_2 or phospholipase C.

3.3 Analysis of control and phospholipase-treated microtubule proteins by gel electrophoresis.

Although the results in Table 7 showed that the inhibition of microtubule assembly by phospholipases was not reversible by soya bean trypsin inhibitor, it was decided to analyse samples from turbidity experiments for protein degradation by SDS-PAGE. As can be seen from Figure 17, there was a marked qualitative reduction in the

TABLE 8

The effect of phospholipases on microtubule assembly in the presence of sodium fluoride.

ADDITIONS	% CONTROL ± S.D.			
	ΔOD	p	R	p
Phospholipase A_2	80.6 ± 4.0	<0.05	80.4 ± 4.6	<0.05
Phospholipase C	75.4 ± 5.7	<0.05	63.8 ± 13.7	<0.05

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2 - 3 $mg.ml^{-1}$, were preincubated with 0.5 $units.ml^{-1}$ phospholipase C or 0.22 $units.ml^{-1}$ phospholipase A_2 for 15 minutes at 37°C in the absence of GTP. Microtubule assembly was initiated by the addition of 0.5mM GTP and optical density (350nm) recorded for 15 minutes in a thermostatted spectrophotometer cell housing. Buffer conditions were identical to the experiment described in Table 7 except for the inclusion of 10mM NaF. From the recorded spectrophotometer traces the initial rate (R), after termination of the lag period, and the full extent of microtubule assembly (ΔOD) were calculated. Results are expressed as a percentage of control samples ± S.D. from three independent experiments. Paired 't' tests were used, to assess statistical significance (p) compared to control samples, as indicated.

FIGURE 17

Electrophoretic analysis of phospholipase-treated microtubule proteins.

After completion of the turbidity experiments described in Figures 23 and 25, one-cycle-purified microtubule proteins were freeze-dried. They were then resuspended and boiled in an equal volume of electrophoresis sample buffer, which contained 2% SDS, and then separated on a 4 - 15% polyacrylamide horizontal slab gel with a 4% stacking gel (except gels a and b which had a 10 - 15% gradient with a 10% stacking gel). Gels a, b, e and f contained 5 - 10 µg of microtubule protein whereas gels c,d,g and h were overloaded with 50 - 70 µg. Samples represent typical gels (one of duplicate gels from three separate experiments with each phospholipase) and are either control (C), phospholipase A_2-treated (PLA) or phospholipase C-treated (PLC) microtubule proteins.

The major band in each gel is tubulin. A reduction in the levels of MAPS 1 and 2 can be seen in samples incubated with PLC(gels f and h).

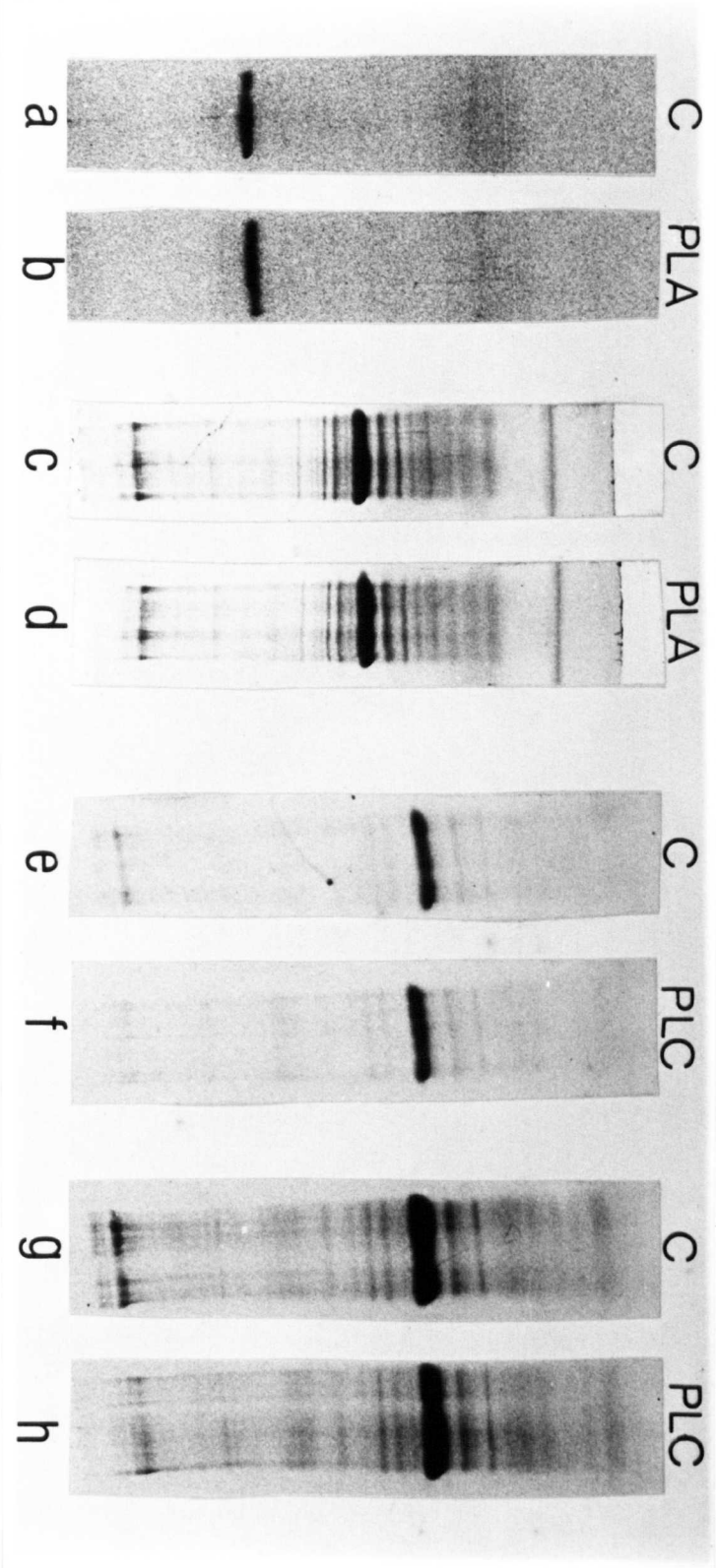


FIGURE 17.

levels of the high molecular weight doublet polypeptides (MAPs 1 and 2) as a result of incubation with phospholipase C (compare gels e and g with gels f and h, respectively). In contrast, phospholipase A_2 caused no detectable changes in the levels of the same proteins (compare gel c with gel d). MAPs were not detectable in gels a and b (control and phospholipase A_2 treatment) at all. This was probably due to the fact that in these cases a 10 - 15% polyacrylamide gradient was used (compared to a 4 - 15% gradient in gels c - h) which may have prevented the MAPs from penetrating the gel during the time course of electrophoresis.

When the gels were scanned by a densitometer there was no detectable change in the electrophoretic profile as a result of phospholipase A_2 treatment (Figure 18; traces a - d). Phospholipase C treatment resulted in the selective reduction of MAPs 1 and 2, but produced no detectable change in tubulin or tau polypeptides. Degradation of MAPs by phospholipase C was accompanied by an increase in the densitometric peak area of a polypeptide with a molecular weight of approximately 100,000 (Figure 18; traces e - h).

These results suggested that phospholipase A_2 did not contain a detectable protease activity. However, in the case of phospholipase C there was a contaminating protease which selectively degraded MAPs 1 and 2, which are known to be required in the regulation of microtubule assembly <u>in vitro</u> (Herzog and Weber, 1978; Murphy and Borisy, 1975). So it was probable that the protease

FIGURE 18

Densitometric analysis of polyacrylamide gels of phospholipase-treated microtubule proteins.

The gels shown in Figure 17 were scanned with a Zeiss mark I densitometer. Typical densitometer recordings are shown of control and phospholipase-treated microtubule proteins. The letters on the traces refer to the corresponding gel in Figure 17. Tubulin , MAPs and tau polypeptides are indicated in addition to a polypeptide band which increased in peak area as a result of phospholipase C treatment (100K).

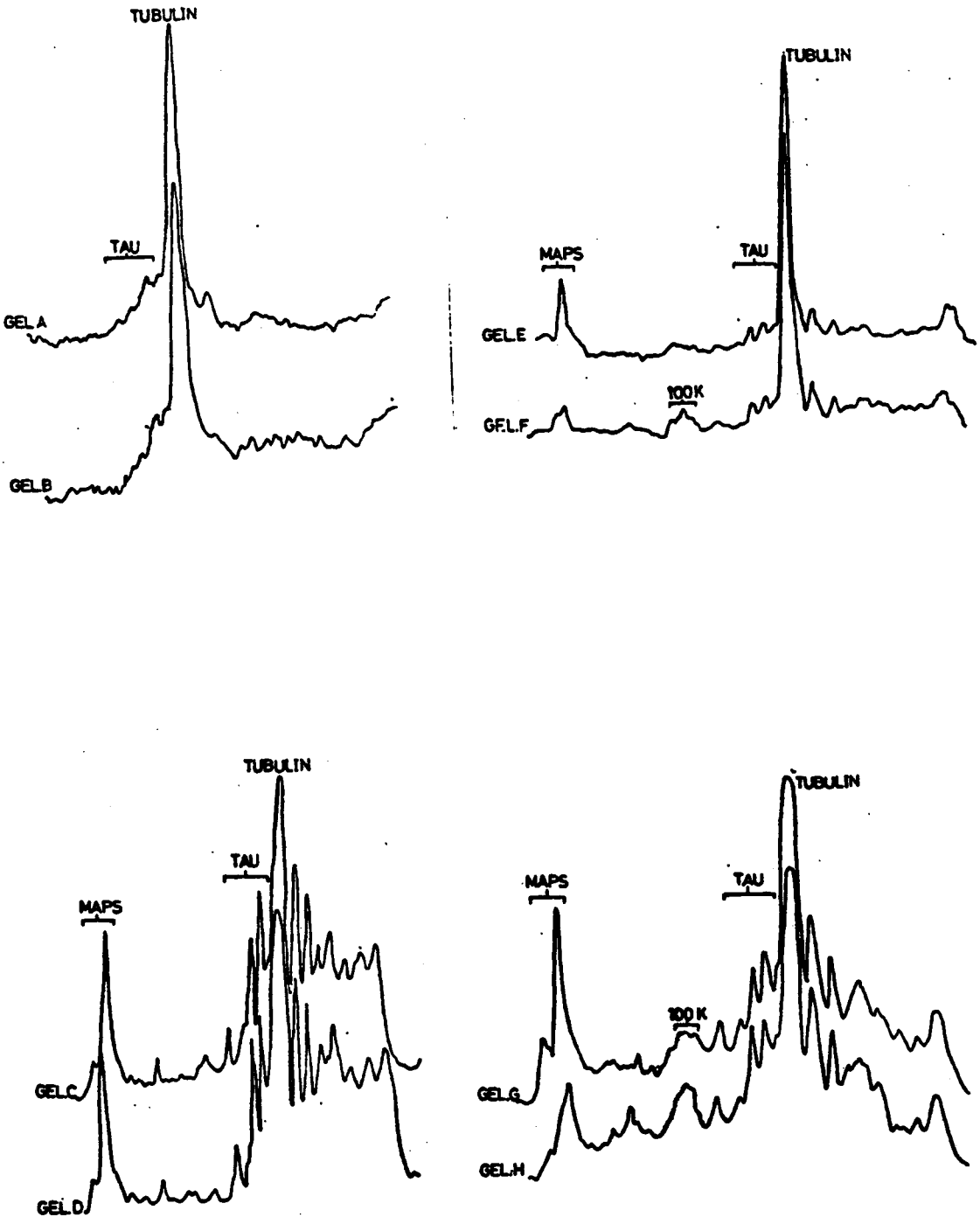

TUBULIN
TAU
GEL A
GEL B
TUBULIN
MAPS
TAU
GEL E
100K
GEL F
TUBULIN
TAU
MAPS
GEL C
GEL D
TUBULIN
MAPS
TAU
100K
GEL G
GEL H

FIGURE 18.

contaminant present in phospholipase C preparations masked the true effects of phospholipid hydrolysis on microtubule assembly.

Such findings underline the problem of the purity of commercial enzyme preparations. It may also be that the levels of contaminants may vary from one batch to another. It must be considered of utmost importance to check each batch for relevant contamination and, where possible, to purify the enzyme further before using it in experiments which may be particularly sensitive to specific contaminating enzyme activities.

3.4 Analysis of control and phospholipase-treated microtubules by electron microscopy.

Specimens, stained directly with 1% uranyl acetate, showed no apparent difference in morphology from controls whether treated with phospholipase A_2 or phospholipase C (Figure 19). This suggested that the modified phospholipids were involved in the nucleation or elongation of microtubules, but had no effect on microtubule morphology. It may be that modification of phospholipids is unlikely to have major effects on the size or shape of microtubules and that such changes may be difficult to detect by this method.

Presumably, the degradation of MAPs, as a result of phospholipase C treatment (Figures 17 and 18) reflected a reduction in the number of microtubule side-arm projections which are known to be largely composed of MAPs (Herzog and Weber, 1978). However, these projections are not at all visualised by this method of staining.

FIGURE 19

Electron microscopic analysis of the effects of phospholipase A_2 on microtubule morphology.

After completion of turbidity development of the samples described in Tables 7 and 8, samples were taken for electron microscopy and stained directly, on collodion-coated grids, with 1% aqueous uranyl acetate. Shown are three typical electron micrographs (one from four experiments) of (a) control and (b) phospholipase A_2-treated and (c) phospholipase C-treated microtubules.
Magnification = 100,000.

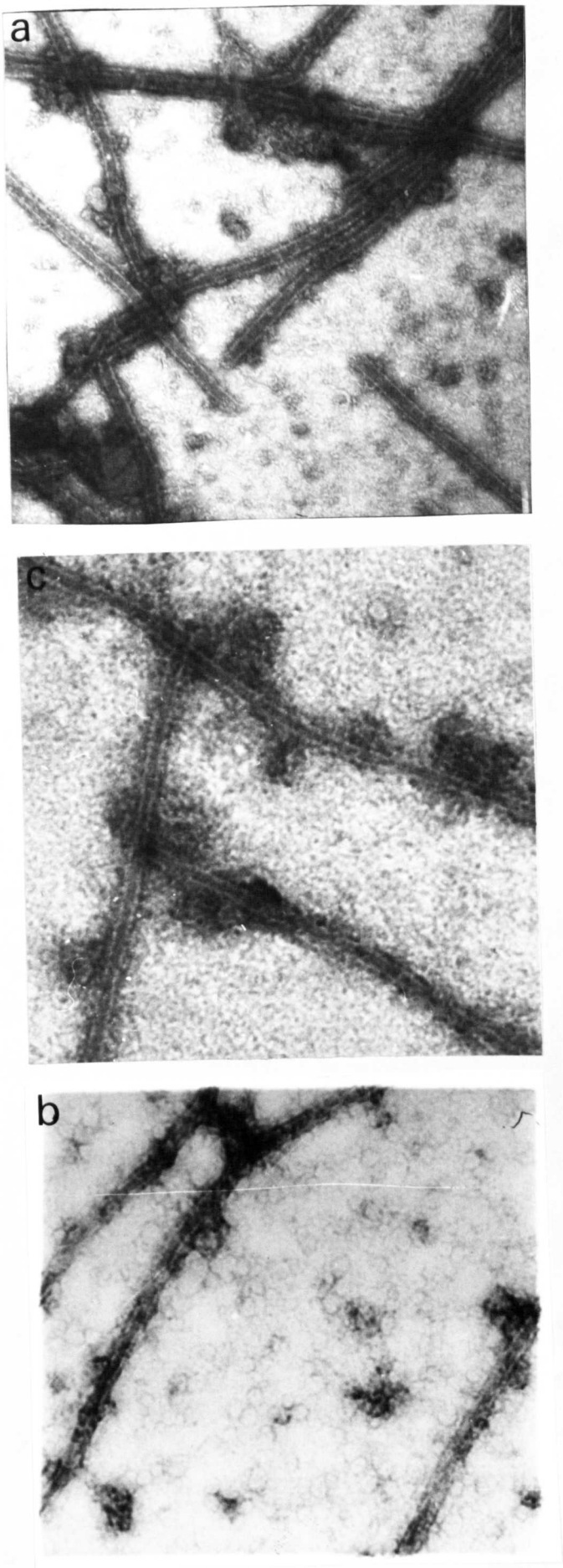


FIGURE 19.

3.5 The effects of exogenous phospholipids on microtubule assembly.

Having established that a number of phospholipids were associated with highly-purified microtubule proteins (Results Section 2), and that microtubule assembly was inhibited by exogenous phospholipases (Results Section 3.1 and 3.2), it was decided to investigate the possibility that exogenous phospholipids might influence microtubule assembly. These experiments concentrated on two of the major microtubule-associated phospholipids PC, PE and their lysophospholipids (LPC and LPE). The amount of phospholipid added in each experiment (25 - 30 μM) was roughly equivalent to one to two-fold greater than the amount of total phospholipid phosphate associated with one mg of three-cycle-purified microtubule proteins.

A sonicated aqueous suspension of each exogenous phospholipid was added to various concentrations of microtubule protein prior to microtubule assembly. PE increased the extent of microtubule assembly at all protein concentrations (Table 9), but at two concentrations its effect, although consistent, was only approaching significance ($p<0.05$). This was probably due to the amount of variation in a small number of experiments. However, if paired 't' tests were carried out on data pooled from all eleven experiments, then the effect was significant. The effects of PC were inconsistent and varied with protein concentration. Such differences may be due to the ratio of exogenous to endogenous phospholipid which may influence the interaction between a given phospholipid and microtubule

TABLE 9

The effects of exogenous phospholipids on microtubule assembly.

One-cycle-purified microtubule proteins, resuspended in RB at various concentrations as indicated, were incubated at 37°C for 20 minutes in the presence or absence of 25-30μM phosphatidyl choline (PC), lysophosphatidyl choline (LPC), phosphatidyl ethanolamine (PE) or lysophosphatidyl ethanolamine (LPE). Phospholipids were added, as 10μl of a concentrated sonicated suspension in ice-cold RB, at zero time (before GTP) and thoroughly mixed by vigorous vortex mixing. Then the initial optical density at 350nm was recorded. Microtubule assembly was then initiated by the addition of 0.5mM GTP and incubation for 30 minutes at 37°C after which the final optical density was measured. Microtubule assembly was calculated as the total change in optical density and the results are expressed as mean $\Delta OD_{350} \pm$ S.D. for three experiments run in parallel at each protein concentration (except only two experiments carried out at 0.4mg.ml^{-1}). Statistical significance of the results was estimated by paired 't' tests at each protein concentration and on data pooled from all protein concentrations.

TABLE 9

PROTEIN CONCENTRATION ($mg.ml^{-1}$)	CONTROL ΔOD	PE ΔOD	p	LPE ΔOD	p	PC ΔOD	p	LPC ΔOD	p
1.6	0.071 ±0.005	0.093 ±0.002	<0.05	0.065 ±0.006	NS	0.074 ±0.014	NS	0.065 ±0.014	NS
1.2	0.029 ±0.003	0.059 ±0.010	0.1>p>0.05	0.026 ±0.005	NS	0.053 ±0.008	<0.05	0.033 ±0.005	NS
0.8	0.007 ±0.002	0.014 ±0.004	0.1>p>0.05	0.007 ±0.005	NS	0.010 ±0.0	0.1>p>0.05	0.009 ±0.002	NS
0.4	not detected	0.004 ±0.001		not detected		not detected		not detected	
SIGNIFICANCE OF COMBINED RESULTS			<0.01		NS		NS		NS

proteins. This would also be consistent with the larger relative changes in the extent of assembly in the presence of PC and PE at the lower protein concentration (Table 9). When the data from all experiments with PC were pooled the overall result was not statistically significant. LPE and LPC had no significant effect on the extent of microtubule assembly.

When experiments were carried out at a protein concentration of $0.4 mg.ml^{-1}$, microtubule assembly was only detectable in the presence of exogenous PE. The presence or absence of microtubules was confirmed by electron microscopy (not shown). This protein concentration is approximately the critical concentration (_i.e._, the concentration of total protein below which microtubule assembly will not occur). These results suggest that PE may be able to nucleate microtubule assembly by lowering the critical concentration.

In summary, the results shown above suggest that PE is able to nucleate or enhance microtubule assembly _in vitro_. The effects of PC were inconsistent, but at low protein concentrations it may enhance microtubule assembly, whereas LPE and LPC had no significant effect at any protein concentration.

4. INTERACTIONS BETWEEN MICROTUBULES AND PURIFIED SMOOTH ENDOPLASMIC RETICULUM IN VITRO.

The data presented in Results Section 2 show that there is a persistent association between a number of phospholipids and microtubules purified *in vitro*. This finding, together with the effects of added exogenous phospholipids and phospholipases on the extent of microtubule assembly *in vitro* (Results Section 3), indicates that some phospholipids may be important in the regulation of microtubule assembly or function. Alternatively the association may reflect a close interaction *in vivo* between microtubules and intracellular membranes. The experiments presented in this Section involved the simulation of such an interaction with smooth endoplasmic reticulum (SER) *in vitro*.

4.1 Assessment of Membrane Purity.

Before the execution of experiments using purified SER it was necessary to assess the purity and integrity of the membrane preparation and also to obtain yields sufficiently high to carry out experiments on the scale required. The yields of SER were about 0.2mg protein per gram wet weight tissue when 15mM CsCl was incorporated into the sucrose gradient centrifugation procedure. Such yields were insufficient to carry out experiments on the scale required. CsCl is used in such preparations to enhance the separation of rough and smooth microsomes by chelating the ribosomes of the rough fraction which results in a higher rate of sedimentation (Depierre and Dallner, 1976). Yields were increased to 1 - 2 mg protein per gram tissue wet weight

when CsCl was omitted from the purification procedure. Such yields were adequate for the experiments to be carried out and also provided a good separation of rough endoplasmic reticulum (RER) from SER, as judged by a 4 - 5 fold enrichment in RER of the specific activity of the endoplasmic reticulum marker enzyme glucose-6-phosphatase (Figure 20). This result was confirmed by electron micrographs of membranes used in subsequent experiments (next Section), which were generally smooth in appearance (Figures 25, 26 and 27b).

The SER membranes were then subjected to further marker enzyme analyses to determine contamination by other sub-cellular components. Monoamine oxidase and cytochrome-C-oxidase were used as markers for outer (Robinson et al., 1967) and inner (Wharton and Tzalgoloff, 1967) mitochondrial membranes respectively, and acid phosphatase was used to estimate presence of lysosomal membranes (Hodges and Leonard, 1974). 5'-nucleotidase was used as the marker for plasma membranes (Morré, 1971) and thiamine pyrophosphatase for golgi apparatus (Allen and Slater, 1961). The level of contamination by a particular membrane was estimated either as the percentage recovery in purified SER of total enzyme activity from a crude homogenate from which the SER was derived, or by a comparison of the specific activities of the marker in purified SER with that found in a purified marker reference. In the latter situation the marker reference was assumed to be of 100% purity, which could lead to an over-estimation of contamination by the marker.

FIGURE 20.

Glucose-6-phosphatase activity in crude microsomal, smooth (SER) and rough endoplasmic reticulum (RER) from rat liver.

Microsomal membranes, freshly-prepared from rat liver, were further separated into SER and RER components by sucrose density gradient centrifugation. A glucose-6-phosphatase assay was then performed on each fraction. Results are expressed as mean specific activity $\pm$ S.E.M. of triplicate assays from the same rat liver preparation. Similar results were found in another experiment using different rats.

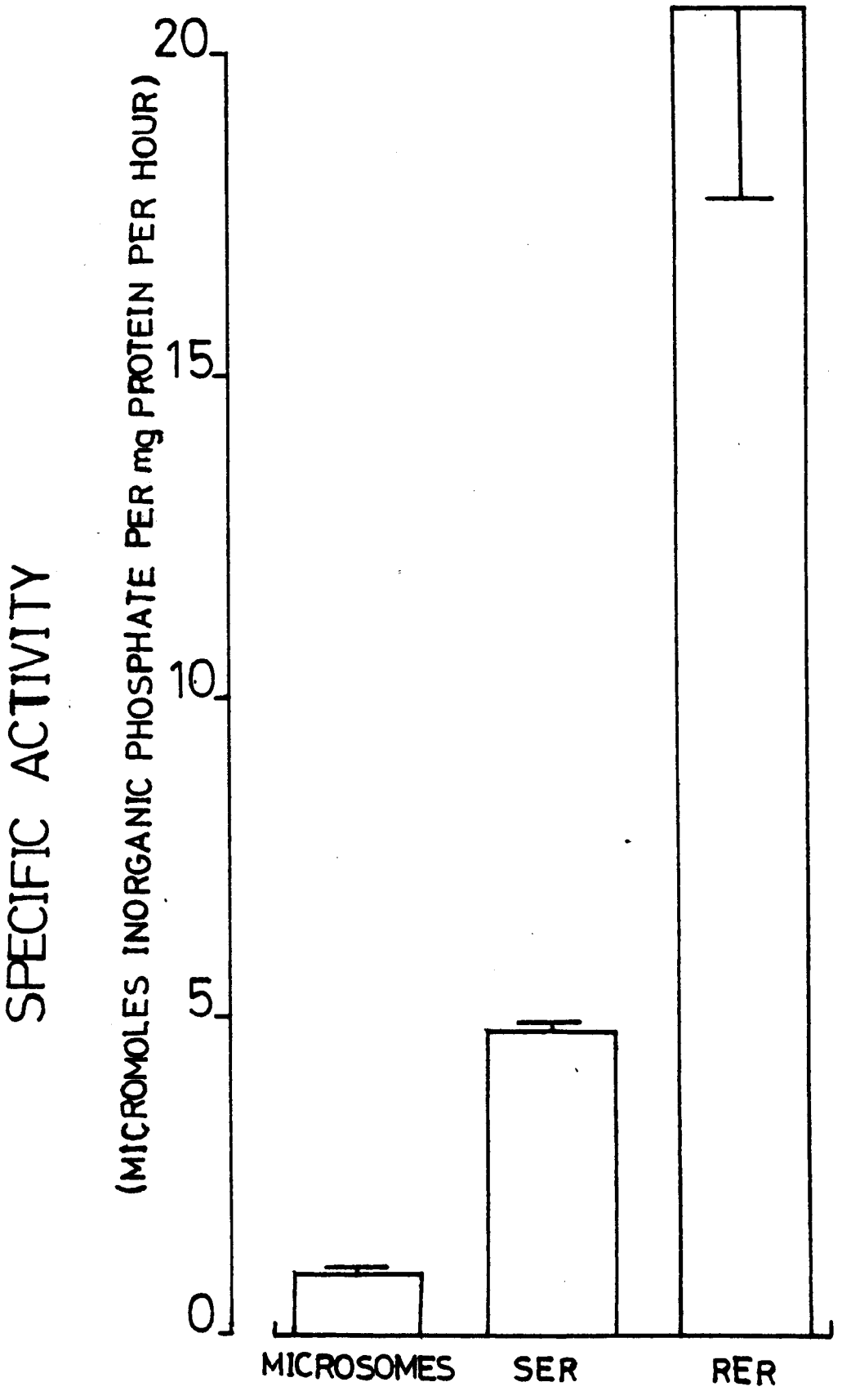

SPECIFIC ACTIVITY
(MICROMOLES INORGANIC PHOSPHATE PER mg PROTEIN PER HOUR)
20
15
10
5
0
MICROSOMES
SER
RER

FIGURE 20.

The data in Table 10 show that the SER preparations used in microtubule-membrane interaction experiments (next Section) contained up to 8% contamination by outer mitochondrial membranes, which could have sheared during homogenisation, and approximately 6% contamination by plasma membranes. Inner mitochondrial membrane marker activity was absent in purified SER and only a slight trace of lysosomal marker activity was detected. The rather high estimate of golgi apparatus levels (~20%) could be misleading, due to the presence of significant levels of an endogenous thiamine pyrophosphatase in rat liver endoplasmic reticulum (Morré et al., 1974). Thus the final estimate of fraction purity at 66% is probably an under-estimate. This is further suggested by the low incidence of structures which resembled the cisternae of golgi apparatus in specimens examined by electron microscopy (Figures 25, 26 and 27b). The absence of mitochondria in the same specimens gave further confirmation of the low cytochrome-c-oxidase activity.

In conclusion, it is shown that the SER preparations are predominantly made up of membranes with smooth surfaces. The preparations contain at least 66%, by protein, SER and a mixture of other smooth membranes such as outer mitochondrial membranes, plasma membranes and golgi apparatus. SER membranes prepared in the same way (Methods 9.3) were used in subsequent experiments (next Section) to simulate an interaction between microtubules and intracellular membranes in vitro.

TABLE 10

Marker enzymes assays to estimate cross-contamination of smooth endoplasmic reticulum by other sub-cellular membrane components.

A crude homogenate, SER and other membrane fractions were prepared from rat livers and assayed, as indicated, for a variety of marker enzyme activities. All assays were performed on freshly-prepared membranes, except monoamine oxidase where mitochondria were frozen and thawed twice before the assay. The level of contamination of SER by a particular membrane was calculated as either the percentage of total enzyme activity (t) of the crude homogenate which was recovered in SER, or direct comparison of specific activity (s) in SER compared to that in a purified marker reference (e.g., cytochrome oxidase, monoamine oxidase and thiamine pyrophosphatase). Specific activities were calculated as μmoles substrate utilised per mg protein per hour and total activities as μmoles substrate utilised per hour. Activities were combined from two assays on independent rat liver extracts and were checked for linearity with time and protein concentration. Each value represents a mean of 4 - 10 determinations. Standard deviations (not shown) were within the range ± 20%.

TABLE 10

MARKER ENZYME	ORGANELLE	SPECIFIC(s) OR TOTAL (t) ENZYME ACTIVITY			% CONTAMINATION
		CRUDE HOMOGENATE	100% REFERENCE	PURIFIED SER	
Acid phosphatase	lysosomes	4220.6 (t)	-	trace (t)	0
monoamine oxidase	outer mitochondrial membrane	-	1.87 (s)	0.14 (s)	7.7
cytochrome-c-oxidase	inner mitochondrial membrane	-	0.84 (s)	0 (s)	0
thiamine pyrophosphatase	golgi apparatus	-	2.16 (s)	0.43 (s)	19.8
5'-nucleotidase	plasma membrane	3057.91 (t)	-	193.28 (t)	6.3
				TOTAL	33.8

4.2 Effects of added membrane material on microtubule assembly in vitro.

When the amount of added membrane material was low (<0.4 $mg.ml^{-1}$ total protein) it was possible to monitor microtubule assembly by turbidity change. However, higher membrane concentrations interfered with the turbidity measurement causing unstable readings. Therefore, in the presence of higher membrane concentrations the extent of microtubule assembly was measured by the colchicine-binding activity of unpolymerized microtubule protein which remained in the supernatant after centrifugation of microtubules and membranes as described in legend to Figure 22.

In five observations from two independent experiments there was a slight (but not significant) decrease in the rates of assembly and disassembly in the presence of added membranes. The extent of assembly was also reduced similarly by 5 - 20% when microtubule protein:membrane protein ratios were between 40:1 and 20:1. A typical turbidity experiment is shown in Figure 21. Lag periods before the onset of turbidity development were usually retarded by 30 - 90 seconds. These effects, although consistent, were not statistically significant due to the small sample sizes. Assembly in the presence of added membranes was totally inhibited in the presence of 40μM colchicine or 2mM $CaCl_2$ added at 37°C before the GTP. This, coupled with the absence of turbidity change in a sample which contained membranes only, suggested that the observed changed in turbidity were due entirely to

FIGURE 21.

Turbidimetric analysis of the effects of low concentrations of added membrane on temperature-reversible microtubule assembly.

One-cycle-purified microtubule proteins, resuspended in ice-cold RB to a concentration of 2 $mg.ml^{-1}$, were incubated at 37°C in the presence of various concentrations (0.02 - 0.2 $mg.ml^{-1}$) of freshly-prepared rat liver SER. Microtubule assembly was initiated by the addition of 0.5mM GTP and incubation at 37°C. Microtubule disassembly was induced by a temperature shift to 4°C. A typical spectrophotometer recording (one from five in two independent experiments) is shown, in which changes in optical density (ΔOD_{350}) were measured at one minute intervals on four samples, run simultaneously, in a thermostatted cell housing operated by a cell programmer. Open circles represent the control sample where no membranes were added; open blocks represent microtubule proteins, plus 0.2 $mg.ml^{-1}$ added SER; closed blocks and closed circles represent microtubule protein with membranes plus 40μM colchicine and membranes only, respectively. Downward arrow indicates point where temperature was shifted from 37°C to 4°C in the cell housing.

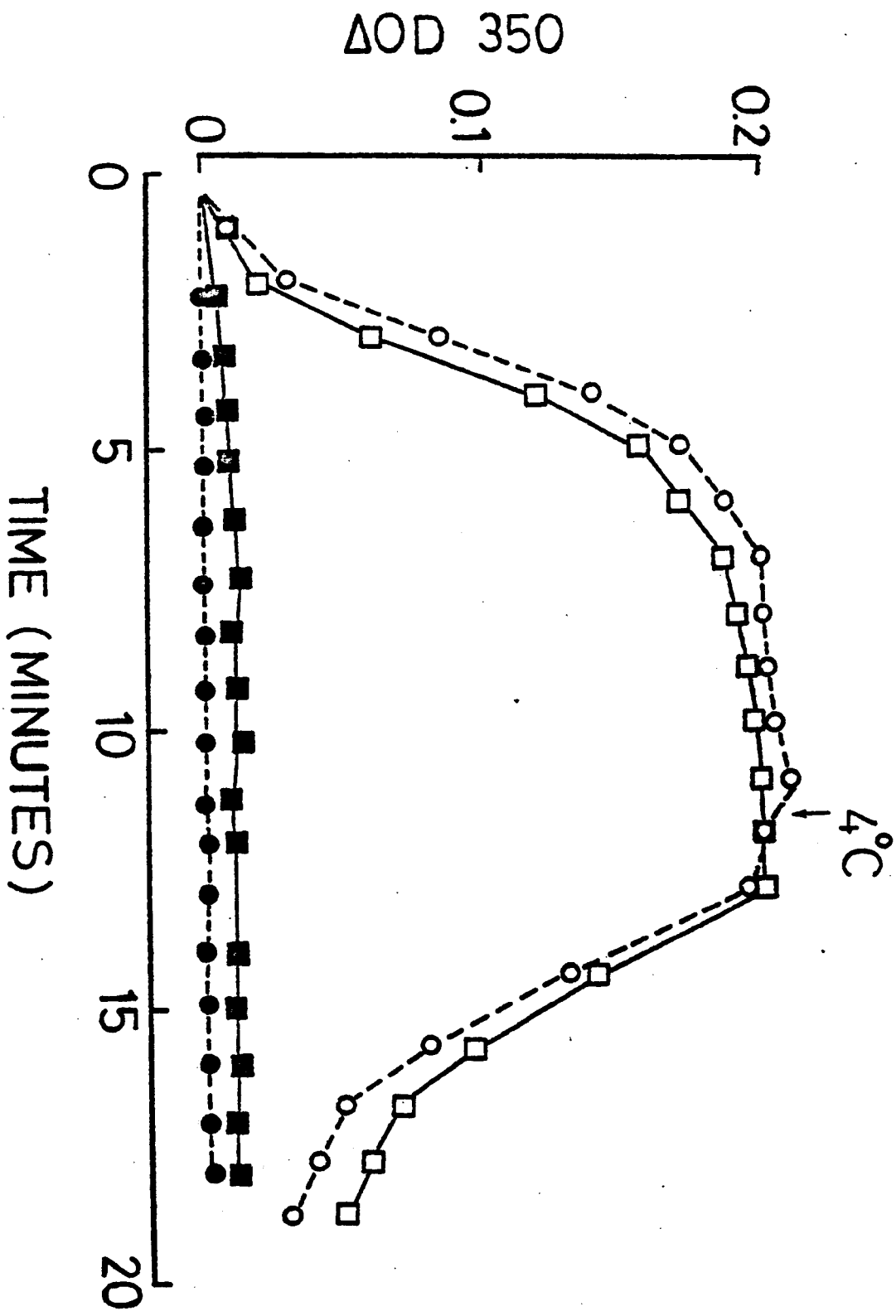


FIGURE 21.

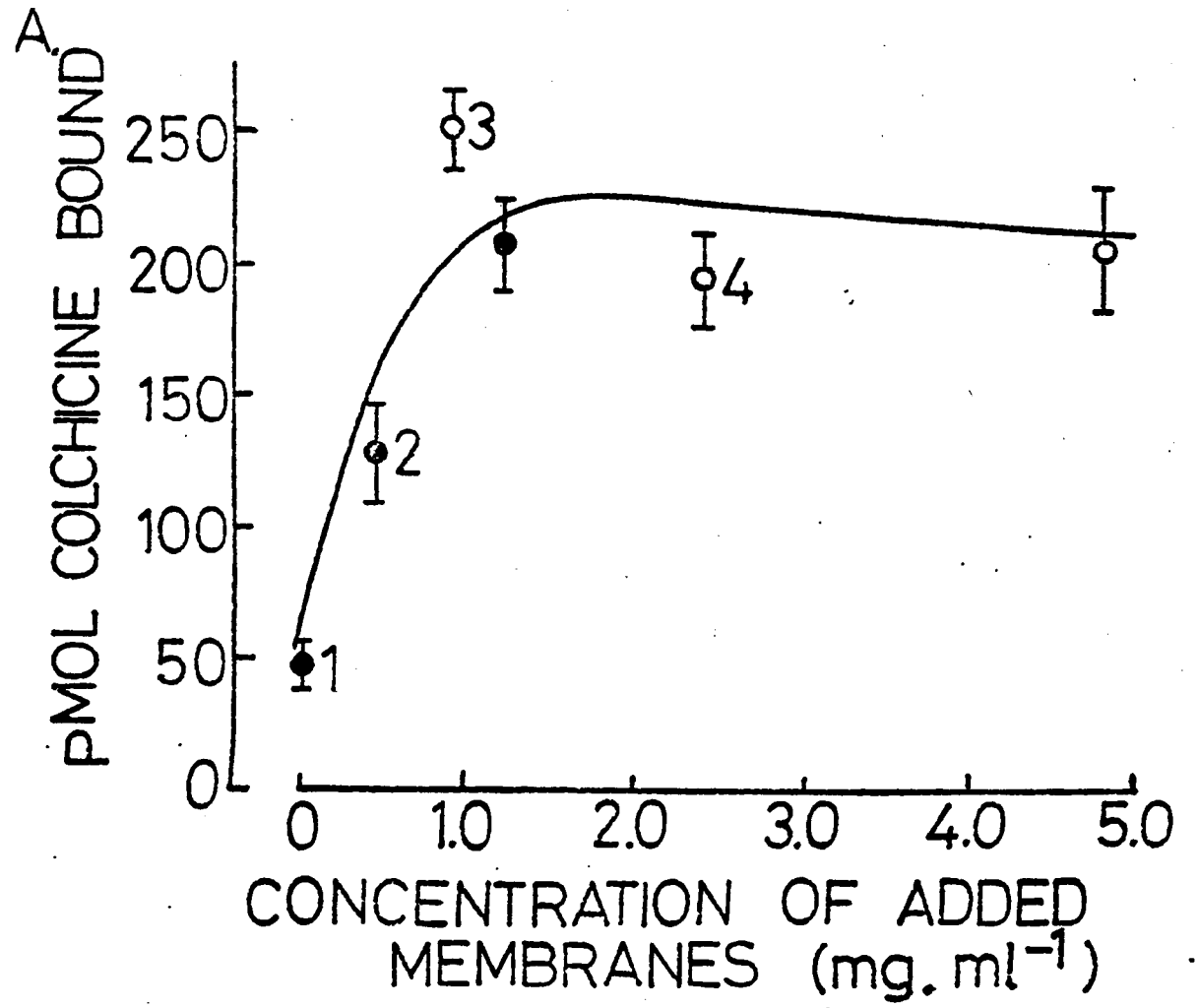
microtubule assembly and that membranes did not interfere in any way with turbidity development at these concentrations (<0.4 $mg.ml^{-1}$). Lower concentrations of added membrane had little or no effect (not shown).

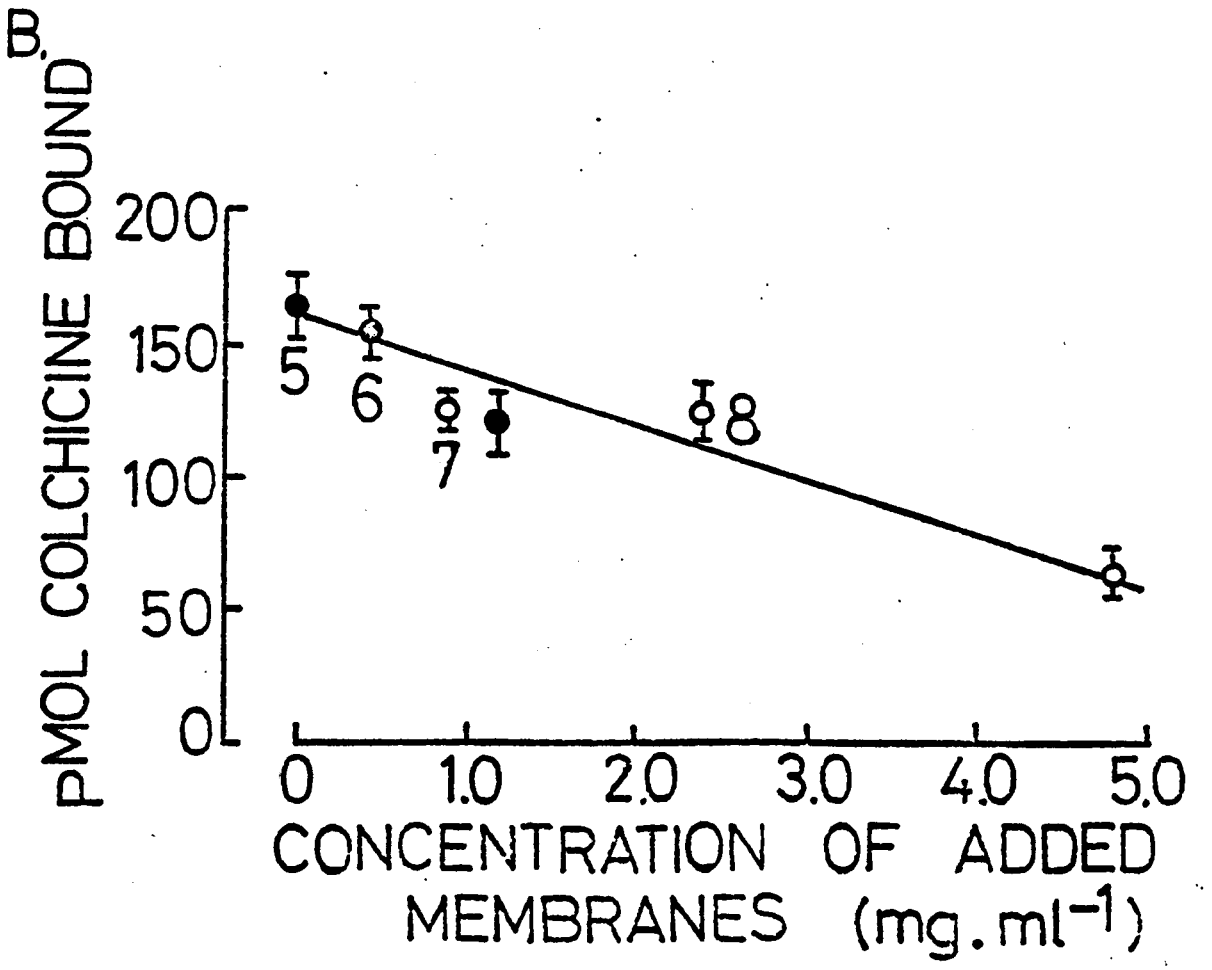
In the presence of higher concentrations of added membrane the extent of microtubule assembly, which was determined by the colchicine-binding activity of unpolymerized tubulin (Borisy, 1972), was inhibited (i.e., colchicine-binding activity was promoted) in the form of a saturation curve (Figure 22a). Maximum effects were observed when the ratio of membrane protein to microtubule protein was approximately 1:2 or above. Colchicine-binding assays were determined, on unpolymerized microtubule proteins, after centrifugation of microtubules and membranes as described in lengend to Figure 22. In the absence of GTP (i.e., non-polymerizing conditions) there was a reduction in the amount of unpolymerized tubulin reflected by a decrease in colchicine-binding activity, as the membrane concentration was increased (Figure 22b). This suggested that some tubulin was either adsorbed on to membrane vesicles or trapped during centrifugation. For this reason a correction factor was introduced to account for tubulin lost in this way under polymerizing conditions (i.e., where GTP was present). The correction factor was calculated as the difference in colchicine-binding activity between the sample where no membrane was added and the appropriate sample where membrane was present. This value was then added to the sample with the corresponding membrane concentration under polymerizing conditions to give the final data point in Figure 22a.

FIGURE 22

The effects of high concentrations of added SER on the extent of microtubule assembly.

One-cycle purified microtubule proteins, resuspended in ice-cold RB to a concentration of 2.4 $mg.ml^{-1}$, were incubated at 37°C for 30 minutes with various concentrations of freshly-prepared rat liver SER as indicated, either in (a) the presence or (b) absence of 0.5mM GTP. Microtubules and membranes were then pelleted by centrifugation at 35,000g for 45 minutes at 37°C. A colchicine-binding assay was then performed immediately on 0.5ml aliquots of the supernatant which contained unpolymerized microtubule proteins. Results are expressed as pmoles of colchicine bound and are a mean ± S.E.M. of 3(o) or 6(•) assays at each membrane concentration. The results of triplicate assays from two independent experiments were combined. The remainder of the supernatant proteins were stored at -70°C for use in assays of colchicine-binding decay (Table 11). Samples numbered 1 - 8 were later examined by SDS-PAGE (Figure 23). Specimens were prepared for electron microscopy after turbidity development for 30 minutes, by direct negative staining, and ultra-thin sections were prepared from pellets after centrifugation (Figures 24 - 27).


A.
pMOL COLCHICINE BOUND
250
200
150
100
50
0
1
2
3
4
0
1.0
2.0
3.0
4.0
5.0
CONCENTRATION OF ADDED MEMBRANES (mg. ml^{-1})


B.
pMOL COLCHICINE BOUND
200
150
100
50
0
5
6
7
8
0
1.0
2.0
3.0
4.0
5.0
CONCENTRATION OF ADDED MEMBRANES (mg. ml^{-1})

Colchicine-binding activity decays with time and is known to be dependent on the presence of GTP and the concentration of protein (Wiche and Furtner, 1980). For this reason it was more necessary to correct all data points for the decay of colchicine-binding activity. Such experiments were repeated on aliquots of supernatant protein, from the experiment described in Figure 22, which had been stored at -70^{o}C for up to six weeks. There was no clear trend in the stability of colchicine-binding activities under the various combinations of added membrane, total protein and GTP concentration which had been involved in the original experiments, (Table 11). Approximately two thirds of all half-lives, whether in the presence of GTP or not, were between four and twelve hours. There were a few samples which produced no decay at all and two others with relatively high half-lives of 15.5 (-GTP) and 17.2 hours (+GTP). There were also considerable differences between corresponding samples from two different experiments. Such differences are difficult to explain as they are probably due to a variety of parameters such as protein concentration, GTP and different microtubule and membrane extracts used in the two experiments. The latter reason seems unlikely when one considers the similarity in the general trends in Figure 22 which, from two experiments, were almost superimposable. Colchicine-binding values obtained in Figure 22 were duly corrected for decay of binding activity during the experimental incubation for the colchicine-binding assay.

TABLE 11

Analysis of colchicine-binding decay of unpolymerized microtubule proteins after microtubule-membrane interactions.

Unpolymerized microtubule proteins, after incubation with various membrane concentrations as indicated in Figure 22, were stored at -70°C for up to five weeks. Colchicine-binding assays were performed in triplicate on 0.5ml aliquots of each sample after a pre-incubation at 37°C, in the absence of colchicine, for 2, 4 or 6 hours. In each sample the equation for the best-fit straight line (for decay of colchicine-binding with time) was calculated by linear regression. From these data the values for half-lives of colchicine-binding were estimated, as indicated. Using half-life values the colchicine-binding data shown in Figure 22 were modified accordingly for decay which occurred during the colchicine-binding assay at a single-time-point (i.e., over four hours incubation in the presence of colchicine). The results are compiled from two experiments from which the concentrations of added membrane protein and of soluble protein (not sedimented by centrifugation) are also shown.

TABLE 11

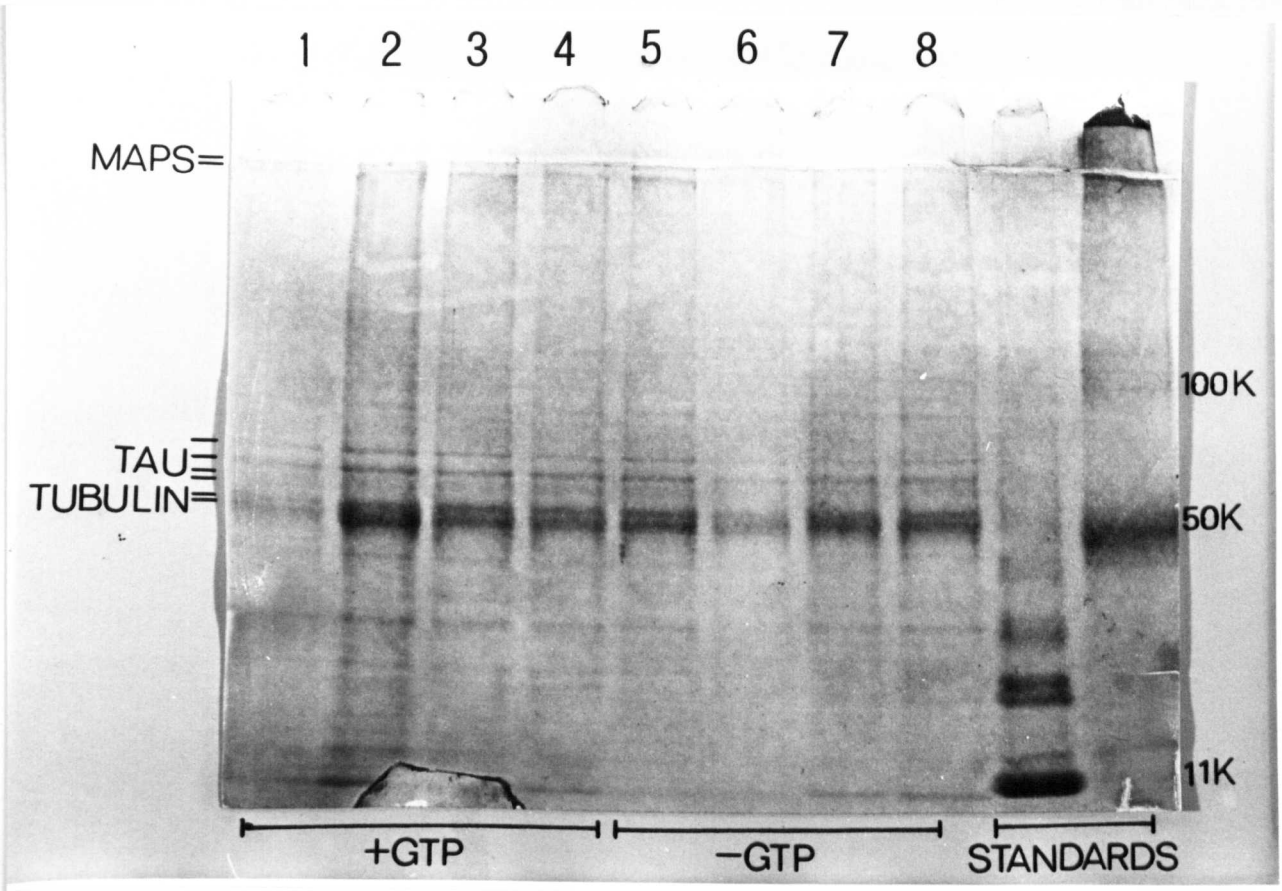
CONCENTRATION ADDED SER ($mg.ml^{-1}$)	CONCENTRATION OF SOLUBLE PROTEIN ($mg.ml^{-1}$)		EXPERIMENT (I or II)	HALF-LIFE OF COLCHICINE-BINDING	
	+GTP	-GTP		+GTP	-GTP
0	1.0	2.5	I	7.8	5.8
	0.8	2.75	II	6.8	11.9
0.5	1.7	1.83	I	5.9	8.2
	1.37	1.57	II	no decay	15.5
0.9	2.1	1.9	I	5.9	8.2
1.2	2.4	2.3	I	5.7	5.4
	1.95	1.75	II	17.23	no decay
2.4	1.7	2.1	I	6.3	3.9
4.8	2.8	2.2	II	10.7	no decay

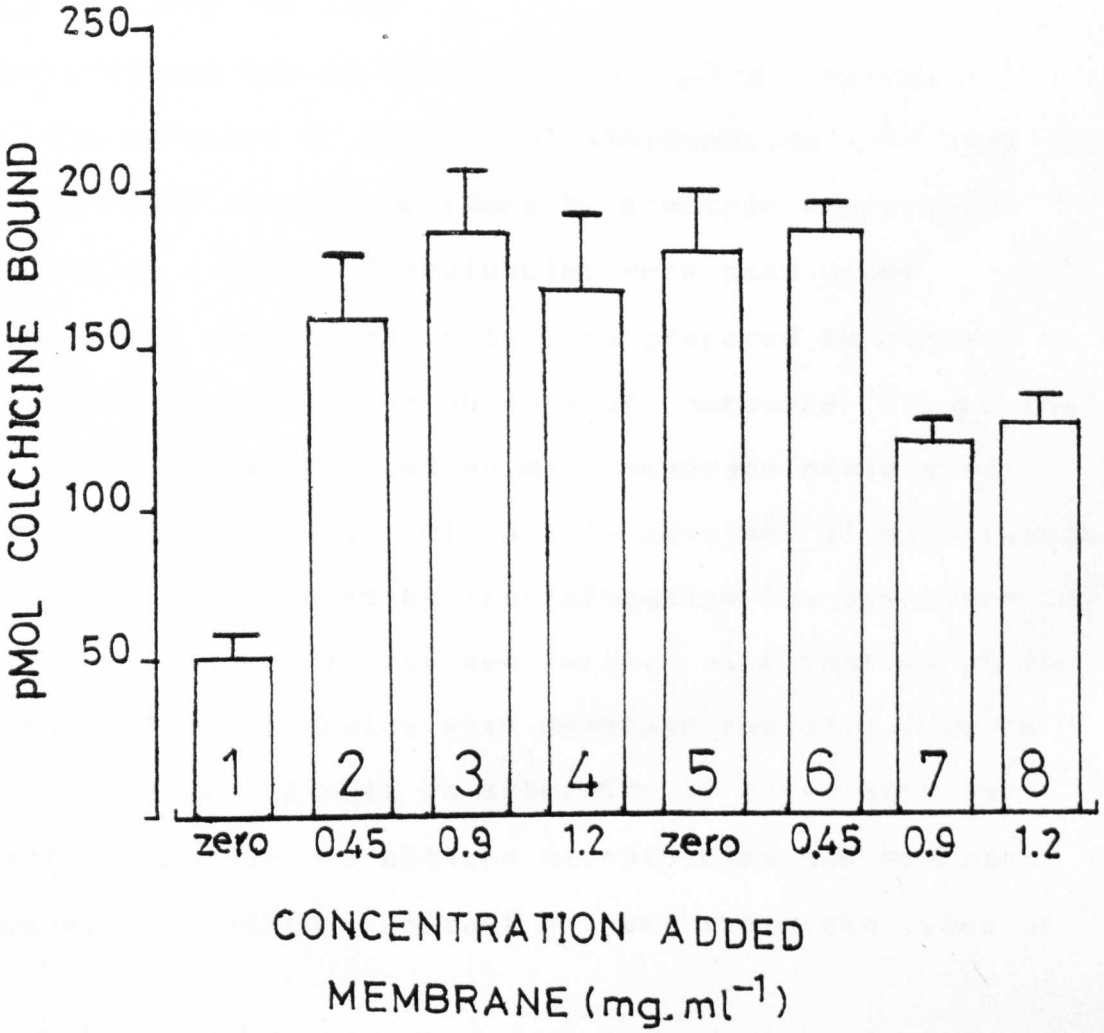
The results described above indicate that smooth intracellular membrane material is able to influence the dynamic equilibrium between polymerized and non-polymerized microtubule protein. One possible explanation for how this occurs might be the adsorption of microtubule nucleation factors such as MAPs (Murphy and Borisy, 1975) and tau (Cleveland et al., 1977) by membrane vesicles. This possibility was examined by analysis of aliquots of non-polymerized microtubule protein, taken from the experiment described in Figure 22, by SDS-PAGE. The data presented in Figures 23a and 23b show the relationship between colchicine-binding activity of non-polymerized microtubule proteins and their corresponding protein profile. The changes in tubulin visualised by electrophoresis were similar to the changes in their appropriate colchicine-binding values,whether GTP was present during the experiment or not. In the presence of GTP without added membrane there were relatively low levels of all microtubule proteins. In the presence of membranes there was an increase in levels of tubulin by electrophoresis and colchicine-binding. Polypeptides with molecular weights corresponding to the microtubule associated proteins MAPs 1 and 2 (Murphy and Borisy, 1975) and tau (Cleveland et al., 1977) also increased although the MAPs components did not reach their corresponding levels shown in depolymerized microtubule protein where membranes were absent (Figure 23a; compare gels 2 - 4 with gel 5). This suggested that some MAPs may have been adsorbed onto the membranes during the incubation of microtubules with membranes. In the absence

FIGURE 23.

Electrophoretic analysis of unpolymerized microtubule protein after microtubule-membrane interactions.

Samples of unpolymerized microtubule protein from the experiment described in Figure 22 (samples 1 - 8) were freeze-dried, resuspended and boiled in electrophoresis sample buffer and then separated on a 4 - 15% polyacrylamide gradient gel with a 4% stacking gel. Equal volumes of microtubule protein from the original experiment (2 - 10 µg protein) were electrophoresed in parallel to reflect the exact changes in colchicine-binding activity which are shown in the histogram below each sample. Colchicine-binding for samples incubated with membranes in the presence of GTP (+GTP) were not corrected for tubulin losses due to adsorption onto membranes. Two gels which contained low and high molecular weight standards, as indicated, were also electrophoresed in parallel.


1 2 3 4 5 6 7 8
MAPS=
TAU
TUBULIN
100K
50K
11K
+GTP
−GTP
STANDARDS


250
200
150
100
50
pMOL COLCHICINE BOUND
1 2 3 4 5 6 7 8
zero 0.45 0.9 1.2 zero 0.45 0.9 1.2
CONCENTRATION ADDED
MEMBRANE (mg. ml^{-1})

of GTP, there was an initial decrease in the levels of all microtubule proteins (Figure 23a; compare gels 5 and 6) in the presence of added SER. However, the levels of tubulin, or a protein of identical molecular weight, recovered at higher concentrations of added membrane and so did the levels of tau protein. In contrast, MAPs did not recover on the addition of higher membrane concentrations (Figure 23a; compare gel 6 with gels 7 and 8). This observation, together with the observed partial recovery of MAPs in the presence of GTP at higher concentrations of added membrane, suggested that there was a selective adsorption of MAPs onto membranes which was inhibited by GTP. Although the gels described in Figure 23 were only run once, three other experiments which involved the same preparation at higher protein concentrations (up to 80µg) showed similar trends.

The presence or absence of microtubules with membranes was confirmed in all specimens by electron microscopy (Figures 24 - 27). Microtubules were seen under polymerizing conditions in samples prepared by direct negative staining in the absence of membrane (Figure 24a) and in samples where 0.45 $mg.ml^{-1}$ membrane protein was present (Figure 24b). Ultra thin sections of microtubules and membranes pelleted by centrifugation (as described in legend to Figure 22) provided further confirmation of the presence of microtubules with membrane vesicles (Figure 25). It was difficult to determine if there were any specific associations between microtubules and membrane vesicles as, although contact between these two types of

FIGURE 24

Electron micrographs of microtubules polymerized in the presence or absence of SER.

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2.4 $mg.ml^{-1}$, were incubated with or without membranes at 37°C for 30 minutes in the presence of GTP. Samples of 5µl volume were stained directly on collodion-coated copper grids with 1% aqueous uranyl acetate. Shown are two typical electron micrographs of microtubules polymerized in (a) the absence or (b) the presence of 0.45 $mg.ml^{-1}$ of freshly-prepared rat liver SER. Magnification 6,300. The large black object in (b) is probably a membrane fragment.

In the presence of membranes(b) the microtubules are shorter and more branched in appearance than control samples(a) although no attempt was made to quantify these differences. Such changes correspond to the reduction in turbidity development of microtubule preparations incubated with membranes (Figure 21).

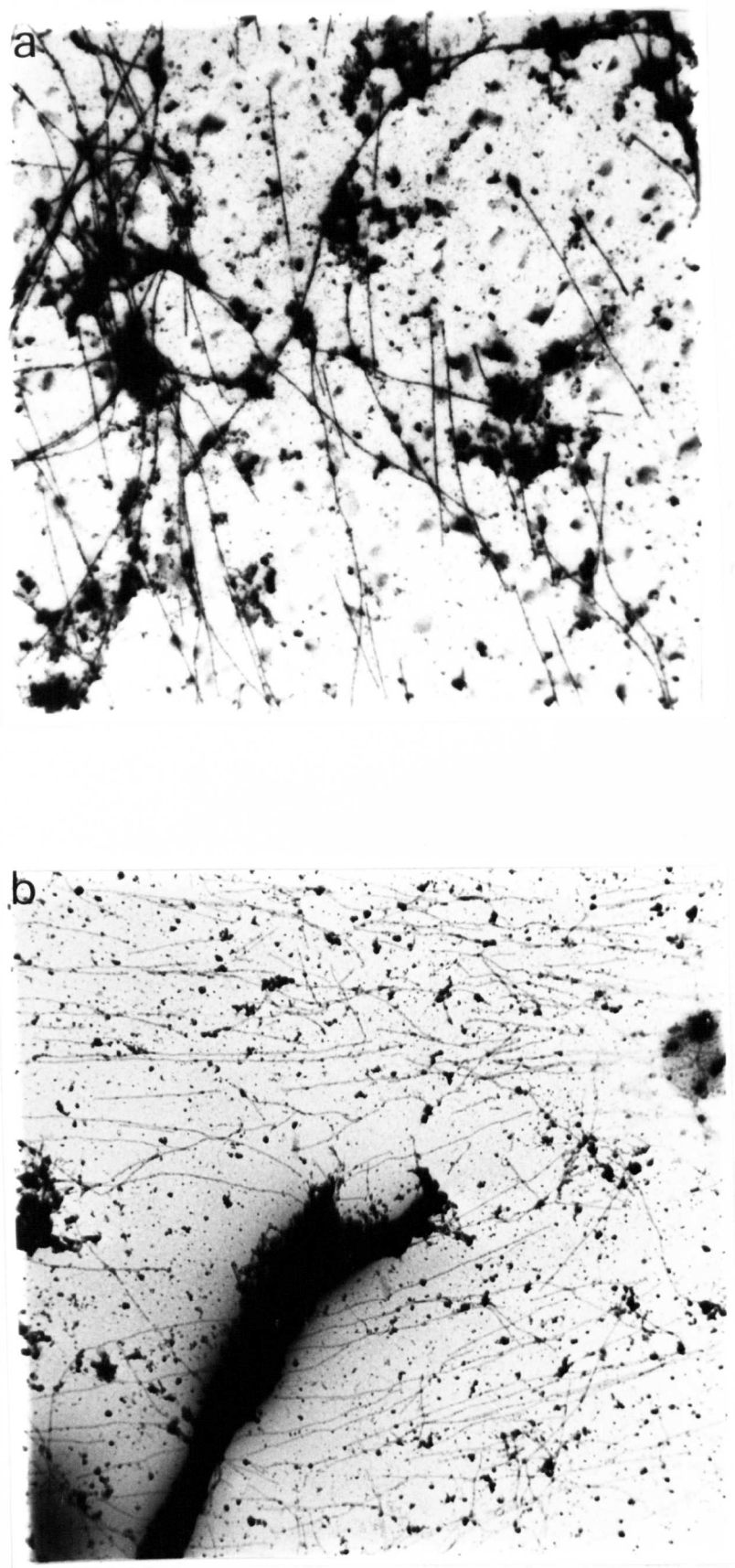


FIGURE 24.

FIGURE 25.

Electron micrograph of an ultra-thin section of microtubules polymerized in the presence of SER.

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2.4 $mg.ml^{-1}$, were incubated with 0.45 $mg.ml^{-1}$ added SER at 37°C for 30 minutes in the presence of GTP. Microtubules and membranes were pelleted by centrifugation at 35,000g for 45 minutes at 37°C. Pellets were fixed, dehydrated and embedded in epon using a standard procedure. Silver-gold sections were cut with a glass knife, stained with aqueous uranyl acetate and then with lead citrate. Shown is a typical electron micrograph (from one of two experiments) in which both microtubules and membrane vesicles are present. Magnification 100,000.

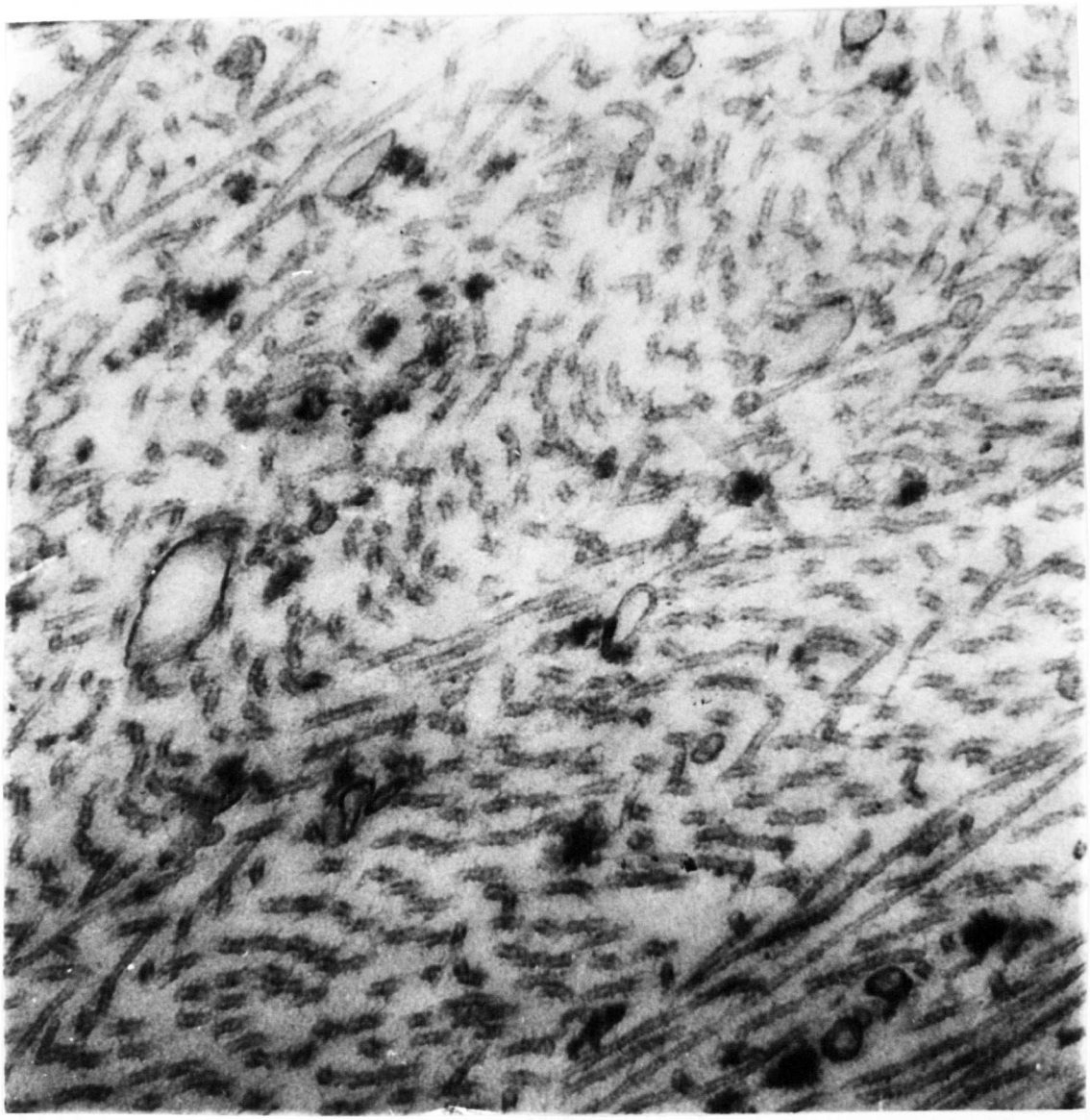


FIGURE 25.

organelle were observed (Figure 25), such associations may have developed as a result of the centrifugation process. It would be necessary to carry out a much more detailed statistical analysis of serial sections to quantify the significance of these associations. At higher concentrations of added membrane, in the presence of GTP, microtubules were not observed either by direct negative staining (Figure 27a) or in ultra-thin sections (Figure 27b). However, the close packing of membrane vesicles after centrifugation, made the observation of any microtubules almost impossible in ultra-thin sections (Figure 27b). As expected, microtubules were never detected in samples where GTP was absent. In these cases the only structures observed were protein aggregates and (when added) membranes (Figure 26).

In summary, the findings from the work in this Section suggest that smooth intracellular membrane material is able to influence the dynamic equilibrium between polymerized and non-polymerized microtubule proteins <u>in vitro</u>. The inhibition of microtubule assembly by the SER preparations may have been caused by the adsorption of MAPs on to membranes with a corresponding reduction in the number of microtubule nucleation sites <u>in vitro</u>.

FIGURE 26

Electron micrograph of microtubule proteins after incubation in the presence of SER under non-polymerizing conditions.

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2.4 mg.ml^{-1}, were incubated in the presence or absence of 0.45mg. ml^{-1} of freshly-prepared rat liver SER at 37°C for 30 minutes in the absence of GTP. Membranes were then pelleted by centrifugation at 35,000g for 45 minutes at 37°C. Pellets were then fixed, dehydrated and embedded in epon by a standard procedure. Silver-gold ultra-thin sections were then cut with a glass knife and stained with aqueous uranyl acetate and then lead citrate.Shown is a typical electron micrograph (from one of two experiments); magnification 100,000.

The structures shown are mostly membrane vesicles and amorphous protein aggregates. The very small circular structures (arrows) correspond in size to tubulin rings.

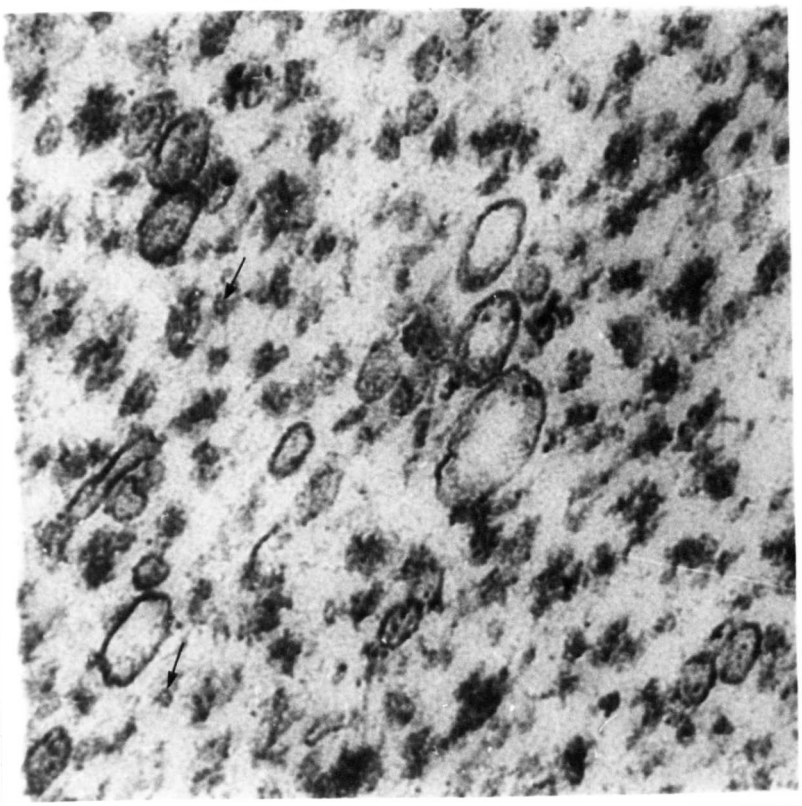
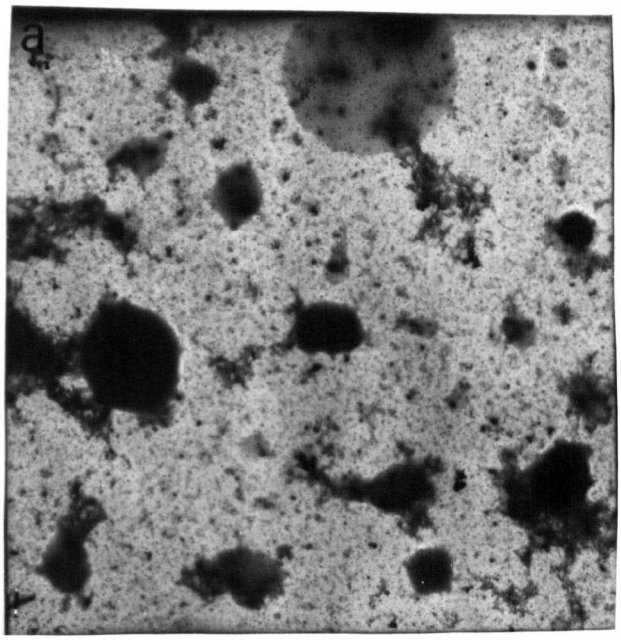


FIGURE 26

FIGURE 27

Electron micrographs of microtubule proteins after incubation with high concentrations of SER in the presence of GTP.

One-cycle-purified microtubule proteins, resuspended in RB to a concentration of 2.4 $mg.ml^{-1}$, were incubated with 2.4 $mg.ml^{-1}$ of freshly-prepared rat liver SER at 37°C for 30 minutes in the presence of GTP. At this point a 5µl specimen was applied to a collodion-coated copper grid and stained directly with 1% aqueous uranyl acetate (a). Microtubules and membranes were then pelleted by centrifugation at 35,000g for 45 minutes at 37°C. Pellets were fixed, dehydrated, etc., using a standard procedure. Silver-gold sections were then cut with a glass knife. Shown are typical electron micrographs (from one of two experiments) of (a) direct negative-stained specimen; magnification 6,300 (b) ultra-thin section; magnification 40,000. Microtubules were not present in any specimens under these experimental conditions.



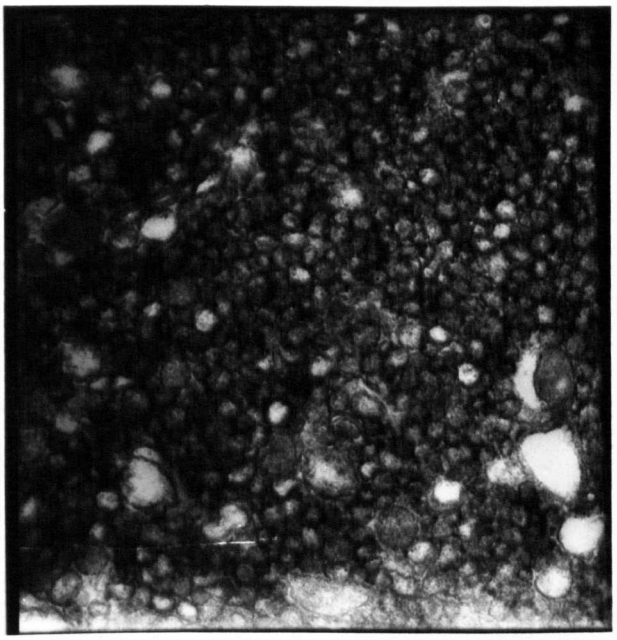


FIGURE 27.

DISCUSSION

SDS-PAGE analysis of microtubule proteins isolated by temperature-dependent recyclization showed that purified microtubule extracts consisted of at least twenty polypeptides (Figure 6). The major components, identified by their apparent molecular weight, were tubulin, MAPs and tau which were present at levels comparable to previous reports (Gaskin et al., 1974; Larsson et al., 1976; Borisy et al., 1974; Asnes and Wilson, 1979). Some of the minor accessory protein components, which appeared on overloaded gels, may be responsible for some of the protein/enzyme activities which are found in association with microtubules, but are not attributed to MAPs or tau, such as calmodulin (Marcum et al., 1978) or tubulin-tyrosine ligase (Murofushi, 1980), although no attempt was made to identify such proteins by their molecular weight alone.

The buffer conditions which produced optimum microtubule formation were 100mM PIPES, 1mM EGTA, 0.5mM $MgSO_4$ at pH 6.8 and microtubule assembly was induced by the addition of 0.5mM GTP and incubation at 37°C. These conditions were utilised in all experiments described in RESULTS, unless indicated otherwise.

There was an absolute requirement for Mg^{2+} and optimal microtubule assembly was supported at concentrations of up to 0.5mM added Mg^{2+} (Figure 1). This may reflect the formation of an Mg.GTP complex during microtubule assembly (Arai, Ihara, Arai and Kaziro, 1975). In addition, Mg^{2+} may be required as a cofactor for microtubule-associated enzymes such as Mg^{2+}-activated ATPases (White, Coughlin and Purich, 1980).

In contrast, 0.4mM free Ca^{2+} added before initiation of microtubule assembly reduced the extent of microtubule formation by approximately 50% (Figure 1) - an effect which was not reversible by the subsequent addition of EGTA (which chelates Ca^{2+}). This effect may be attributed to the presence of a Ca^{2+}-activated protease such as that described by Sandoval and Weber (1978). When 2mM Ca^{2+} was added to preassembled microtubules there was an instantaneous depolymerization which resulted in the formation of disc-like structures (Figure 2). This effect was reversible with EGTA and may be due to binding of Ca^{2+} to tubulin (Hayashi and Matsumura, 1975) or a Ca^{2+}-activated calmodulin-like protein (Marcum et al., 1978).

The fact that microtubule assembly was reversible at low temperature and in the presence of ca. 2mM free Ca^{2+} together with the observation that colchicine inhibited microtubule formation are in accordance with previous reports (Olmstead and Borisy, 1973, 1975; Shelanski et al., 1973; Lee and Timasheff, 1975) and indicate the usefulness of these physical and chemical parameters as indicators of the validity of turbidity experiments as a measure of microtubule assembly.

Some of the major aims of the present study were to identify phospholipids associated with microtubules, to quantify their association with highly-purified microtubules and to determine whether specific protein-phospholipid associations existed.

The data presented in Figure 10 demonstrated that there was a consistent pattern of phospholipids in

association with microtubules prepared through up to three cycles of purification. At this stage of experimentation the major phospholipids, which were identified by R_f and specific spray reagents, were identified as PC, PE, PS, PI, SP along with cholesterol and at least four other unidentified components (Figure 7 and Tables 2 - 4 inclusive). Comparison of tlc of one-cycle-purified microtubule-associated phospholipids with those extracted from a crude brain homogenate showed that there were relative differences in the levels of a few phospholipids on charred chromatograms (Figure 9). This suggested that the observed distribution of phospholipid associated with microtubules was not a direct artifact of the fractionation of crude brain homogenate. However, it was not possible to measure individual phospholipids for a more quantitative comparison due to the low levels of microtubule-associated phospholipids present which could not be detected by phosphate determination.

Relative changes in the level of individual microtubule-associated phospholipids over three cycles of purification were measured by densitometry. Such measurements showed that all of the major phospholipids detectable by charring (i.e., PC, PE, PS-PI and SP) decreased to a similar extent as microtubules became more highly-purified (Table 5). The fact that total phospholipd phosphate decreased to a similar extent suggested that no individual component became enriched relative to any other after three cycles of microtubule purification. However, this observation was limited to those phospholipids which were readily

detectable at these levels by charring. It was found, for example, that a number of phospholipids such as LPC and DPG (discussed later) and the unidentified chromatogram components were less sensitive to charring. This may have been due to low concentration or low fatty acyl content.

Fortunately, the levels of total phospholipid were sufficiently high to be quantified by total phospholipid phosphate assay. To justify the use of this method it was necessary to show that the GTP present in microtubule preparations did not interfere with the assay. In such experiments (data not shown), it was established that inorganic phosphate from GTP was not detectable in chloroform:methanol extracts from RB containing GTP. Presumably the GTP was removed in the aqueous phase of the phospholipid extraction procedure.

Although the levels of total phospholipid phosphate decreased significantly between one and three cycles of microtubule purification (Figure 11), the subsequent enrichment at five cycles indicated that the phospholipids were unlikely to be due to non-specific contamination from membrane fragments. If this were the case then the phospholipid content would be expected to continue to decrease relative to protein through successive cycles of purification. The fact that the phospholipid content became enriched at five cycles of microtubule purification may be due to a higher affinity of the phospholipid for polymerized rather than depolymerized microtubule protein and may reflect kinetic changes in highly-purified microtubules.

It is also possible that phospholipid levels are affected by proteolytic and lipolytic enzyme activities present in the brain tissue during the time interval between slaughter of the animal and microtubule extraction. However, the fact that electrophoretic and thin-layer chromatogram patterns were quite reproducible from different brain extracts and the small range of variation in total phospholipid content of three-cycle-purified microtubules (values pooled from two separate brain extracts) suggested that such effects were not significant in highly-purified microtubules. However, one source of variation in the levels of individual microtubule-associated phospholipids could be differences in the diet of the pigs which were reared at a number of local farms (data not available).

Daleo et al., (1977) analysed total phospholipid phosphate associated with rat brain microtubule protein and, assuming that all of the phospholipid was associated to tubulin, calculated a level of one mol phospholipid phosphate per twelve tubulin dimers. However, it was not clear from their work whether the microtubules were purified through one or two cycles of temperature-dependent recyclization.

If one makes the same assumption with the microtubule proteins used in the experiment presented in Figure 15, then there could be as much as 1.5mol phospholipid phosphate/mol tubulin dimer at three cycles of purification. (This calculation assumes a molecular weight of 110,000 for the tubulin dimer and that the preparations contain 75%, by weight, tubulin.) This difference may be

explained by the fact that rat brain tubulin is associated with less phospholipid than that from pig brain. However, another possibility is that their method of microtubule preparation, which incorporated glycerol in the assembly buffer (Shelanski et al., 1973), may have been responsible for the lower content of microtubule-associated phospholipid. For example, it may be that glycerol, which forms the backbone of the phospholipid structure, is able to interact with lipid binding sites on microtubule proteins in such a way as to displace microtubule-associated phospholipids. Evidence to support this view was obtained by Kirazov and Lagnado (Personal communication 'a'), who studied the incorporation of ^{32}P into chick brain microtubule-associated phospholipids. After in vivo injection of label they subsequently purified microtubule proteins in the presence and absence of glycerol from a common pool of brain homogenate. They found that the levels of total ^{32}P-labelled microtubule-associated phospholipid were five-fold greater when microtubules were purified in the absence of glycerol.

Now that the presence of a significant amount and consistent pattern of phospholipid in my microtubule preparations had been established, it was necessary to determine whether these molecules were associated with tubulin or its accessory proteins.

It was in fact shown that tubulin and its accessory proteins, purified by phosphocellulose chromatography, contained approximately equal amounts of phospholipid

phosphate relative to total protein (Table 6). For tubulin this represented a level of approximately two moles phospholipid phosphate per mole tubulin dimer. The observed stoichiometry would not be inconsistent with the fact that such phospholipids may be of importance in the regulation of tubulin-tubulin interactions during microtubule formation.

Striking differences were observed in the distribution of phospholipids associated with the tubulin and accessory protein fractions (Figures 13a, 13b and 13c), which may reflect specific functions for certain microtubule-associated phospholipids. It appeared that most of the phospholipids present in unfractionated microtubule protein extracts were also present in association with the accessory proteins. However, there were relatively few phospholipids present in the tubulin fraction. These included LPC, along with a spot which had an R_f similar to the DPG standard, and two unidentified components, one of which contained a free amino group. The fact that a sonicated suspension of phospholipid standards were distributed quite differently on phosphocellulose chromatography (Figure 15) confirmed that the observed associations were real and not caused by some interaction between microtubule protein-associated phospholipids and the phosphocellulose resin.

The presence of DPG in association with tubulin is an interesting observation. This phospholipid is thought to be present almost exclusively in mitochondria and has been used as a cytochemical marker (6th International Methodology Forum, 1979), (Morre, 1979).

The fact that DPG was the only phospholipid detected when low concentrations of total phospholipid from tubulin extracts were chromatographed, suggested that DPG was the major phospholipid associated with tubulin. Whether this is a specific functional association, or whether it reflects contamination by mitochondrial membrane fragments, remains to be determined.

LPC had not previously been detected in chromatograms of one-, two- and three-cycle-purified microtubule-associated phospholipids. This could have been due to a combination of higher amounts of total phospholipid chromatographed and chromatography over a smaller distance which reduced spreading of components from the phosphocellulose fractionation studies. However, it is also possible that degradation of PC to LPC could have occurred during the extensive dialysis which followed fractionation of microtubule proteins on the phosphocellulose columns. This does not alter the conclusion that the distribution of phospholipids between tubulin and its accessory proteins was different, but it could mean that the chromatogram patterns observed (particularly in the case of purified tubulin where LPC was a major component), were not an exact reflection of the actual phospholipid composition.

Kirazov and Lagnado (Personal communication 'b') observed a similar distribution of phospholipid associated with one and two-cycle-purified chick brain microtubule preparations. These workers also found that a considerable proportion of the ^{32}P-labelled microtubule-associated phospholipid, recovered after <u>in vivo</u> ^{32}P administration,

was phosphatidyl inositol. Further analysis of this chromatogram component, which co-migrated during tlc with PS in a similar manner to that described in the present study, showed that it also contained diphosphatidyl inositol. This result, together with the detection of phosphatidyl inositol phosphodiesterase in microtubule preparations (Quinn, 1975) and the observed regulatory effects of myoinositol on microtubule assembly *in vitro* (Kirazov and Lagnado, 1977), suggests that phosphoinositide turnover may be in some way involved in microtubule assembly.

The inositol phospholipids are of particular interest in cellular functions in nervous tissue (Hawthorne and Pickard, 1979). The 4- and 5-phosphate groups on the inositol ring of triphosphoinositide have a rapid turnover and an affinity for divalent cations. The turnover of phosphoinositides is known to increase in response to the entry of calcium ions into iris muscle (Abdel-Latif, Akhtar and Hawthorne, 1977) and synaptosomes (Griffin and Hawthorne, 1978) and there is also evidence to suggest that the inositol lipids may regulate the amount of bound calcium in erythrocyte membranes (Buckley and Hawthorne, 1972). The findings discussed above, together with the presence of microtubules (Hajos and Csillag, 1976; Hajos, Csillag and Kalman, 1979) and membrane-bound tubulin (Zisapel *et al.*, 1980) in synaptosomes, suggest that the turnover of phosphoinositides in conjunction with intracellular calcium levels may be important in the regulation of microtubule-associated phenomena within the cell.

In my experiments the PS-PI chromatogram spot was not examined for the presence of polyphosphoinositides; the main reason for this is that the inositol lipids occur in trace amounts in brain tissue (ca. 3% of total lipid). Using the methods available it was not possible to scale up the microtubule preparations sufficiently to produce enough PS-PI for further analysis. In addition, it has been suggested that the higher phosphoinositides are not completely extracted by the methods used in this thesis and they require a much more rigorous extraction under acid conditions at 37°C (Kai and Hawthorne, 1966). A sensitive method of detection such as the autoradiographic procedure described by Lagnado and Kirazov (1975) would also be essential to analyse the low levels of polyphosphoinositide present.

Taken together all of the experimental findings discussed above suggest that specific associations exist between microtubule proteins and phospholipids. However, the exact nature and function of such associations remains to be determined. It could be that tubulin and/or some of its accessory proteins are lipoproteins. Alternatively, the phospholipids (or, at least, some of them) may take the form of small vesicles distributed along the microtubules. The former possibility is more likely as one would expect membrane-like vesicles to be removed during centrifugation at 4°C, in the temperature-dependent recyclization procedure, unless they had a very low equilibrium density. Furthermore, phospholipid vesicles were not evident from electron microscopy of microtubules.

Having established the presence of a consistent pattern of phospholipids in microtubule preparations it was necessary to discover their possible functions. I concentrated on only two aspects, *viz.* the effects of exogenous phospholipases and phospholipids, to determine whether the enzymic modification or changes in relative levels of individual phospholipids influenced microtubule assembly *in vitro*.

There had been previous conflicting reports in the literature with respect to whether exogenously added phospholipase C promoted (Daleo *et al.*, 1977) or inhibited (Bryan, 1975) microtubule assembly *in vitro*. It was therefore necessary to perform similar experiments in the present study in an attempt to clarify the situation. The data presented in Results Section 3 show that the pre-incubation of microtubule proteins with commercial preparations of phospholipase A_2 and phospholipase C caused, in both cases, a reduction in the initial rate and the extent of microtubule assembly (Figure 25). A partial reversal of inhibition by phospholipase A_2 was brought about on the addition of a PC analogue during the pre-incubation. This suggested that the effects of phospholipase A_2 on microtubule assembly were due at least partly to its effect on a microtubule-associated PC molecule. This partial, rather than complete, reversal of phospholipase A_2 effects on microtubule assembly may indicate that insufficient inhibitor was added to effect total reversal of enzyme action. The amount added (20μM) corresponded to approximately one fifth of the concentration of total

phospholipid phosphate associated with the one-cycle-purified microtubules used in these experiments and may have, therefore, been lower than the PC concentration. Under the experimental conditions employed it was not possible to add a more highly concentrated analogue solution due to difficulties which were encountered in its solubility. As expected, there was no reversal of phospholipase C effects by the PC analogue which was probably due to the fact that phospholipase C has a different site of action (from phospholipase A_2) with which the PC analogue is unable to compete.

Analysis of microtubule-associated phospholipids from control and phospholipase C-treated microtubule proteins showed that phospholipase C had reduced several phospholipids with, surprisingly, the production of PA (Figure 16). Although this finding indicated that the effects of phospholipase C were due to the modification of microtubule-associated phospholipids, electrophoretic analysis of phospholipase C-treated microtubule proteins showed that there had been a reduction in the levels of the accessory proteins MAPs 1 and 2 (Figures 17 and 18). As there was no reduction of tubulin or other accessory proteins and the fact that phospholipase A_2 caused no detectable changes in any microtubule proteins, it was concluded that phospholipase C preparations contained a contaminating protease selective for MAPs. As MAPs are essential for microtubule assembly *in vitro* (Murphy and Borisy, 1975; Sloboda *et al.*, 1976), this side-effect probably masked the true effects of phospholipase C-induced phospholipid modification.

This possibility is supported by the fact that Daleo et al., (1977) used phospholipase C from the same source, but were unable to detect a contaminating protease. In their experiments there was an increase in both the rate and extent of microtubule assembly. The same workers also found that phospholipase A_2 inhibited and phospholipase D promoted microtubule assembly. In their experiments Daleo et al., (1977) found that Ca^{2+} was required to enhance the action of phospholipases in contrast to my own work, where preincubation with Ca^{2+} was shown to inhibit microtubule assembly in the absence of phospholipases. This may reflect differences in the electrophoretic profile of their microtubule preparations due to purification in the presence of glycerol or that glycerol stabilized their preparations against Ca^{2+}-induced inhibition of microtubule assembly. Unfortunately such a comparison is not possible as these workers did not publish their electrophoretic data. Other workers (Bryan, 1975; Nagle and Bryan, 1975) have found that microtubule assembly was inhibited by phospholipase A_2 and phospholipase C, the former being reversible in the presence of a PC analogue, but in neither case were the enzyme preparations checked for a contaminating protease.

As mentioned earlier, one effect of phospholipase C on microtubule-associated phospholipids was the production of PA. This was a surprising observation as the normal product should be a diglyceride and a phosphoryl polar head group. A similar observation was made by Daleo et al., (1974) who attributed this effect to the presence

of a diglyceride kinase activity in microtubule preparations. These authors went on to characterise this enzyme activity and found that it differed from a kinase activity present in the crude soluble protein supernatant from which the microtubules had been derived (Daleo et al., 1976). This observation, together with the presence of a phosphatidyl inositol phosphodiesterase in association with microtubule preparations (Quinn, 1975), suggests the presence of a specific pool of phospholipid-metabolising enzymes which may be important in the regulation of microtubule-mediated phenomena in vivo.

Having established in the present study that phospholipase A_2 and phospholipase C inhibited microtubule assembly, the resultant microtubules were examined for morphological changes by electron microscopy. The fact that no obvious morphological changes resulted from incubation with phospholipase A_2 (Figure 19) suggested that the effects of this enzyme were concerned with the nucleation and/or elongation processes of microtubule assembly. The reduction of MAPs by phospholipase C presumably led to a decrease in number of microtubule side-arm projections of which MAPs are a major component (Herzog and Weber, 1978), although no quantitative analysis was possible because the direct negative staining procedure used in the present study did not show these structures clearly (Figure 19).

In order to determine how and which phospholipids were important in microtubule assembly, the effects of exogenous preparations of some of the major microtubule-associated phospholipids PE and PC and their lysophospholipids

(LPE and LPC) on microtubule assembly *in vitro* were studied.

A sonicated suspension of PE increased the extent of microtubule assembly at a number of protein concentrations. PC was less consistent in its effects in that it enhanced assembly at only two intermediate protein concentrations. These results, together with the observation that LPE and LPC had no significant effect on microtubule assembly, suggested that specific phospholipids may influence microtubule assembly *in vitro*. It could be that the hydrophobic interactions involved in microtubule assembly are enhanced in the presence of diacyl phospholipids (PE and PC), whereas the monoacyl phospholipids (LPE and LPC) are unable to provide a suitably lipophilic environment. The results of PE and PC experiments are also in accordance with the effects of exogenous phospholipase A_2 (Table 7) where conversion of diacyl to monoacyl phospholipid inhibited microtubule assembly.

When microtubule protein concentration was at 0.4mg.ml^{-1} (*i.e.*, less than the critical concentration, below which microtubule assembly will not occur) microtubule assembly was only detectable in the presence of PE. This suggested that at least one phospholipid (PE) was able to nucleate microtubule assembly. The fact that accessory proteins are necessary to stimulate microtubule assembly *in vitro* (Herzog and Weber, 1978) and that microtubule-associated PE is associated exclusively with accessory proteins (Figure 13) is consistent with the idea that the endogenous PE molecule may have a similar function.

The fact that the microtubule protein preparations used in the above experiments still contained endogenous phospholipids may have had a strong influence on the results obtained. For example, the lipid binding sites on microtubule proteins may already be saturated and inhibit the ability of some exogenous phospholipids to exert an effect. Furthermore, some exogenous phospholipids may be more readily exchangable with endogenous molecules. Also, the structural arrangement of the aqueous suspensions of exogenous phospholipids (ie., micelles or liposomes) may vary and this could affect the ability of a phospholipid to interact with microtubule proteins.

Daleo et al., (1977) found that LPC in their experiments caused a significant reduction in the rate and extent of microtubule assembly, although it was used at a much higher concentration (viz., 0.18mg per mg protein) than in the present study. Such a high LPC concentration may have been necessary to overcome the stabilizing effect of glycerol which binds to microtubule protein in the purification procedure which they used (Detrich et al., 1976). The same workers also found that cardiolipin caused a dramatic decrease in the extent of microtubule assembly to approximately 20% of control values with only a slight decrease in the rate of assembly. PC, a phospholipid extract from their microtubule proteins., and a diglyceride 1,2-Diolein produced no significant effects on microtubule assembly (< 5%) whereas 1,2-Dipalmitin increased both the rate (10%) and the extent (30%) of assembly. These findings, together with the effects of

exogenous phospholipids and phospholipases in the present and other studies (Daleo *et al.*, 1977) and the presence of phospholipid-metabolising enzymes in association with microtubule proteins (Daleo *et al.*, 1974; 1976; Quinn, 1975) are strong indications that metabolism of a pool of microtubule-associated phospholipids may be important in the regulation of microtubule assembly. and,therefore, microtubule-mediated processes.

Although much of the data discussed so far suggests that phospholipids could be involved in the regulation of microtubule assembly, this is not the only possible interpretation. The same results could reflect a functional interaction between microtubules and intracellular membranes. Of course the two interpretations are not necessarily mutually exclusive and both could occur *in vivo.* To investigate the possibility of membrane-microtubule interactions, which may be important in the regulation of microtubule-mediated processes within the cell, I simulated such an interaction *in vitro* between rat liver smooth endoplasmic reticulum (SER) and microtubule proteins (RESULTS Section 4). It was necessary to use smooth intracellular membrane material because of the possibility that RNA (from ribosomes) might inhibit microtubule assembly (Bryan, Nagle and Doenges, 1975). The membranes were prepared from rat liver because it is a rich source of microsomal membranes and also because much of the documented evidence concerning ER membrane preparation and marker enzymes refers specifically to rat liver. In addition, there was the possibility that SER prepared

from pig brain might be significantly degraded during the time elapsing between slaughter of the animal and the membrane fractionation, whereas rat liver provided a convenient and fresh source of SER on site.

The major problem encountered in SER preparation was to obtain sufficient yields to perform experiments on the scale required. The method of preparation used was based on that described by Depierre and Dallner (1976) which involved separation of RER from SER by discontinuous density gradient centrifugation in the presence of CsCl. However, I found that the yield of SER was enhanced two to three fold if CsCl was omitted and the time of centrifugation extended (see METHODS 9.2 for further details). The specific activity of glucose-6-phosphatase was four to five-fold higher in RER compared to SER fractions (Figure 20), which suggested that good separation was achieved (Depierre and Dallner, 1976). It was confirmed that this separation was effective when the membranes observed by electron microscopy were smooth in appearance (Figures 25 - 27).

Further marker enzyme assays were performed to assess the level of cross-contamination by other sub-cellular membranous components. The results shown in Figure 30 suggested that there was no contamination by lysosomal membranes (acid phosphatase) or intact mitochondria (cytochrome C oxidase). However, the combined estimate of contamination by plasma membranes (5'-nucleotidase), outer mitochondrial membranes (monoamine oxidase) and golgi apparatus (thiamine pyrophosphatase) was approximately 35%. The major contaminant appeared to be the golgi

apparatus (ca. 20%). The marker enzyme used in this case was thiamine pyrophosphatase which was suggested by Morré et al., (1974) to be an unsuitable marker for the estimation of cross-contamination of rat liver microsomal preparations by golgi apparatus membranes. They argued that the ER contained a significant amount of endogenous thiamine pyrophosphatase activity which would cause an over-estimation of contamination by golgi apparatus. It is possible that the level of contamination for golgi apparatus in my preparations could also be an over-estimation for the same reason. Even if this were the true level of contamination, it is still possible to conclude that the SER preparations used in my experiments contained essentially smooth membranes of which SER was the major component.

The fact that low concentrations of added membrane protein (0.2 - 0.3mg.ml^{-1}) caused a slight, but consistent reduction in the rate and extent of microtubule assembly (Figure 21), suggested that smooth intracellular membrane material may be able to impose constraints on microtubule assembly. One clue as to how this might be achieved was the consistent increase in the 'lag' period of turbidity development which indicated that the presence of membranes interfered with the microtubule nucleation process.

The slight reduction in the rate of microtubule disassembly at low temperature in the presence of membranes suggested that they may also stabilize microtubules in some way. Although these effects were consistent (for

two experiments) they were not statistically significant by students' 't' test mainly due to insufficient data points at the appropriate membrane concentrations. Lower concentrations of added membrane had no detectable effect on turbidimetric measurements of microtubule assembly and higher concentrations interfered with turbidity measurement by producing unstable traces on the chart recorder.

For this reason it was necessary to find an alternative method to measure microtubule assembly in the presence of higher concentrations of added membranes. The colchicine-binding assay described by Borisy (1972) was chosen as the most convenient method for this purpose. Unfortunately it was only possible to measure the extent of microtubule assembly using this method, which required the separation of polymerized from non-polymerized microtubule protein after the experimental incubation was complete (see Methods 3.2). Depolymerized microtubule protein was then determined by its colchicine-binding activity and the extent of microtubule polymerization determined by the changes in colchicine-binding activity in the presence of different amounts of added membranes.

Higher concentrations of added membranes (> 0.4mg. ml^{-1}) increased colchicine-binding activity and hence inhibited the extent of microtubule assembly. A saturation effect was observed when the ratio of microtubule protein to membrane protein was 2:1 (Figure 22). This showed that smooth intracellular membrane material was able to influence the dynamic equilibrium between

polymerized and non-polymerized microtubule protein. The possible mechanisms by which this influence may be exerted are discussed later. Under non-polymerizing conditions (i.e., in samples where GTP was absent) the reduction in levels of colchicine-binding activity suggested that tubulin was adsorbed during incubation on to membrane vesicles, although there was some apparent recovery of colchicine-binding activity at higher membrane concentrations (Figure 22). A correction factor was introduced to account for tubulin lost from the supernatant protein in this way.

The filter assay used in these experiments involved some differences in buffer conditions compared to the Borisy procedure (1972). Such differences included the presence of low concentrations of RB constituents and the inclusion of 1M sucrose which stabilizes the colchicine-binding site of tubulin against decay with time (Tsé and Doherty, 1980). However, as the experimental approach adopted resulted in different concentrations of total protein and GTP, which are both known to influence the half-life of decay of colchicine-binding (Tsé and Doherty, 1980; Wiche and Furtner, 1980), it was still necessary to determine the stability of colchicine-binding in all samples. In addition, the incubation of tubulin with SER preparations could have had unknown effects on the stability of the colchicine-binding site. Due to the long duration of experiments which required fresh membranes (approximately 27 hours) a single-time-point assay was determined for each sample at the end of an experiment.

The remaining material was stored at -70°C until used to determine the half-life of decay which involved an assay at three different time points between 0 and 6 hours pre-incubation in the absence of colchicine (see legend to Table 11 for details).

The calculated half-lives of colchicine-binding in these experiments appeared to show little relationship to any one experimental parameter. This was possibly due to a combination of the differences in total protein, GTP and membrane concentration in individual samples. It may also be that this method of determination of half-lives lacks precision. The almost random spread of half-life values and total absence of decay in one particular sample (Table 11) may reflect the spread of values which would be obtained if a single sample was assayed in this way on separate occasions. Furthermore the varied results could also reflect differences in the stability of the colchicine-binding sites in individual samples during storage at -70°C. However, despite the doubts concerning the reliability of half-life determinations, the use of such correction factors did not affect the final result, in that the overall trends in colchicine-binding data were still the same.

The presence or absence of microtubules and membranes in the appropriate samples during and after microtubule protein/SER incubations was confirmed by electron microscopy (Figures 24 - 27). This suggested that the colchicine-binding assay had been an effective measure of microtubule assembly in these experiments. Microtubules which were normal in appearance in ultra-thin sections

appeared to be closely associated with membrane vesicles (Figure 25). However, it was difficult to determine how real this association was as some membranes could be simply trapped in the microtubule network during centrifugation. The presence of amorphous masses in the parallel experiment under non-polymerizing conditions (Figure 26) may be due to the occurrence of non-specific protein aggregation as these were not observed in the presence of GTP (Figure 25). The smooth appearance of membranes in Figures 25 and 26 together with Figure 27 (where microtubule assembly was totally inhibited at 2.4 $mg.ml^{-1}$ membrane protein) also served to confirm the biochemical marker enzyme studies discussed earlier.

The colchicine-binding data, although it indicated that interactions had occurred between microtubule proteins and SER, did not provide any information as to the nature of the interaction. One possible explanation was that the membranes inactivated some important accessory proteins such as the higher molecular weight MAPs (Murphy and Borisy, 1975) or the tau factor (Cleveland *et al.*, 1977) thus removing essential nucleation factors for *in vitro* microtubule assembly. This possibility was examined by SDS-PAGE of samples from the same supernatant proteins which had been analysed in the colchicine-binding assays (Figure 23). Changes were observed in the electrophoretic pattern of microtubule proteins as a result of incubation with membranes. The observation of a decrease in the levels of all proteins in the presence of 0.45$mg.ml^{-1}$ membranes, under non-polymerizing conditions, suggested that all

microtubule proteins were to some extent adsorbed onto membranes at this concentration. The fact that tubulin and tau levels recovered as membrane concentration increased but MAPs did not, suggested that MAPs were selectively absorbed by the membranes. This would result in the removal of *in vitro* nucleation sites and contribute to the inhibition of microtubule assembly. The apparent recovery in the levels of tubulin and tau may reflect preference for membrane-membrane or membrane-MAP interactions to membrane-tubulin or membrane-tau interactions at higher membrane concentrations.

In the presence of GTP all proteins increased at 0.45 $mg.ml^{-1}$ added SER, but then fell to a lower level, presumably due to absorption by membranes, as the membrane concentrations increased. Although the MAP component of the gel did not disappear completely, as in the cases where GTP was absent, it was present at a lower level than in the completely depolymerized microtubule proteins in the absence of membranes (Figure 23). Presumably the presence of GTP had reduced membrane-MAP interactions, but the membranes had still removed sufficient MAP to inhibit microtubule assembly. Although these results were obtained from the analysis of the microtubule proteins from only one experiment, they do show that microtubule protein-membrane interactions can occur.

Caron and Berlin (1979) found that liposomes of dimyristoyl phosphatidyl choline selectively absorbed tubulin and accessory proteins under non-polymerizing conditions and in the total absence of divalent cations.

Although their experimental conditions differ from conditions used in the present study, their findings nevertheless make an interesting comparison. They went on to discover that the nature of the interaction was hydrophobic and that liposome-adsorbed tubulin formed extensive intermolecular disulphide cross-bridges. Such interactions resulted in the formation of multi-lamellar structures similar in arrangement to intracellular membranes. These results suggest that interactions between microtubule protein and membrane structures can occur and may influence the organization of the membranes.

However, the fact that MAPs may have been selectively absorbed, whereas tubulin and tau levels recovered at higher membrane concentrations in my experiments, implies that the situation with SER is much more complicated than a simple neutral lipid-protein interaction such as that simulated by Caron and Berlin, (1979). This is, of course, due to the presence of a greater variety of lipids and proteins in intracellular membranes.

Although it was shown that membranes selectively absorbed MAPs, the complete removal of MAPs may not be sufficient to inhibit microtubule assembly completely as tau proteins were still present. It could also be that tau proteins are modified during their incubation with membranes in such a way as to inhibit their capacity to nucleate assembly. Another possibility is that GTP may be utilised by membrane-bound ATPases, which are known to be present in microsomal membranes. Although such enzymes have a relatively low affinity for nucleotides other than

ATP (Dahl and Hokin, 1974) they may be present in sufficient amounts to hydrolyse some of the GTP necessary for microtubule assembly. However, this possibility seems even less likely when one considers that many of the ionic requirements, such as Na^+, K^+, Ca^{2+}, etc,, for such enzymes are absent from the buffer system used in my experiments, (Skou, 1957; Dahl and Hokin, 1974). This could possibly have been confirmed had the SER preparation been assayed for GTPase activity under the buffer conditions used for microtubule-membrane interactions.

The work discussed above demonstrates that smooth intracellular membrane material is able to influence the dynamic equilibrium between polymerized and non-polymerized microtubule proteins *in vitro*. Exactly how this interaction occurs is not known although the presence of typical membrane phospholipids in association with microtubule proteins, together with the apparent adsorption of microtubule proteins by SER, suggests that hydrophobic interactions are involved. It may be that similar interactions are involved in the structural associations between microtubules and intracellular membranes *in vivo* (Smith, 1971; Smith *et al.*, 1975; Smith *et al.*, 1977; Makita and Kiwaki, 1978; Chemnitz and Salmberg, 1978; Dentler, 1981) and in the occurrence of membrane-bound tubulin in synaptic vesicle membranes (Zisapel *et al.*, 1980) and ciliary membranes (Stephens, 1977). Such interactions may influence microtubule-mediated and membrane-mediated phenomena within the cell.

In conclusion, the present study has shown that phospholipids are associated with highly-purified microtubule proteins in a way which is consistent with the possibility that specific protein-phospholipid associations occur. The fact that exogenously added phospholipase A_2, phospholipids and SER affected *in vitro* microtubule assembly in various ways suggests that phospholipids may be involved in the regulation of microtubule assembly and that their presence in microtubule preparations may reflect an interaction between microtubules and intracellular membranes *in vivo*. However, despite these findings, several important questions still need to be resolved.

One of these questions must address itself to the problem of whether or not the presence of phospholipids in microtubule extracts is an artifact of the purification procedure. It could be argued that the observation that the major microtubule-associated phospholipids, visualised by charring, were seen to decrease to a similar extent to each other over three cycles of microtubule purification (Table 5), and the presence of DPG (a major mitochondrial membrane component) in three-cycle-purified microtubule proteins (Figure 13) may reflect contamination by membrane fragments. However, the enrichment in total phospholipid phosphate at five cycles of purification (Figure 11), the qualitative differences between microtubule-associated phospholipids and those of a crude brain homogenate (Figure 9) together with the differences in distribution of phospholipids between tubulin and accessory proteins (Figure 13), all suggest that specific microtubule protein-phospholipid associations can occur. It seems, therefore,

that the question of 'fact or artifact?' will not be fully resolved until the presence of microtubule-associated phospholipids can be demonstrated *in vivo*.

Another question arising from the present study is the arrangement of microtubule-associated phospholipids on microtubule structures. For example, are they evenly-distributed along assembled microtubules, or do they form discrete packages at certain points? Are they in the form of membrane vesicles or do they exist as lipoprotein complexes? The presence of microtubule-associated membrane vesicles seems less likely due to their probable removal with cold-stable protein aggregates during temperature-dependent recyclization. However, the possibility that microtubule proteins could assemble into membrane structures under polymerizing conditions in a similar manner to that described by Feit and Shay (1980), cannot be discounted. One way to study thisproblem would be to examine a representative sample of serial sections of microtubule pellets at various cycles of purification. A correlation between numbers of vesicles and levels of total phospholipid phosphate might be indicative of this being the major form taken by microtubule-associated phospholipids. A further problem during the present study is that of how the phospholipids are involved in microtubule assembly. PE (and possibly PC) were shown to enhance microtubule assembly, whereas LPC and LPE caused no significant effect. However, such studies are hampered by doubts as to whether microtubule-associated phospholipid binding sites are saturated or readily exchangeable with exogenous phospholipids.

One possibility might be to remove phospholipids from microtubules before such experiments. Daleo et al., (1977) accomplished this using a neutral detergent Nonidet P-40 during microtubule purification. However, the observation that Nonidet-treated microtubules had a higher specific viscosity and turbidity than untreated microtubules, but had no effect on the total protein sedimented by centrifugation, suggested that the detergent may have bound to microtubule proteins and interfered with measurements. An alternative to detergent treatment might be reconstitution experiments with phosphocellulose-purified tubulin and accessory proteins with their associated phospholipids. Such experiments might also include the effects of phospholipases and a variety of exogenous phospholipids. The importance of microtubule-associated phospholipids in microtubule assembly could be more clearly established by extending the work in this way.

Further extensions to the present work might be the quantification of individual microtubule-associated phospholipids by phosphate assays of chromatogram components from scaled-up experiments. Accessory proteins could be purified further to examine the specificity of protein-phospholipid associations in more detail. Microtubule preparations could also be analysed quantiatively for the presence of cholesterol and neutral lipids and experiments carried out to determine their importance in microtubule assembly in vitro.

Studies of phospholipid turnover might yield important information as to their function in microtubule assembly.

Such studies could be carried out by the addition of exogenous labelled phospholipids to microtubule proteins under

(i) non-polymerizing conditions

(ii) during microtubule assembly

(iii) at steady state *in vitro*.

Alternatively, the microtubule-associated phospholipids could be pre-labelled either *in vivo* or *in vitro*, with labelled glycerol or ^{32}P, in a manner similar to that described by Lagnado and Kirazov (1975). Such studies could be extended to changes in turnover or metabolism of microtubule-associated phospholipids in the presence of Ca^{2+} and other microtubule inhibitors. Of particular interest in this respect would be the polyphosphoinositides (di-, and triphosphatidyl inositol), which exhibit a rapid turnover in response to Ca^{2+} ions (Hawthorne and Pickard, 1979).

Finally, the distribution of phospholipids along microtubules assembled *in vitro* could be determined using monospecific anti-microtubule-associated phospholipid antibodies to decorate assembled microtubules, followed by electron microscopic analysis. The main problems in such a study would probably be the specificity of such antibodies, which are likely to cross-react with other microtubule-associated phospholipids. This problem would be even more difficult in an *in vivo* situation due to the abundance of phospholipids in membrane structures. However, antibodies against microtubule-associated DPG and PI, which are generally less widely distributed in brain tissue, may be successful. Such a study *in vivo* would be essential to prove that microtubule-associated phospholipids are not an artifact of the preparation procedure.

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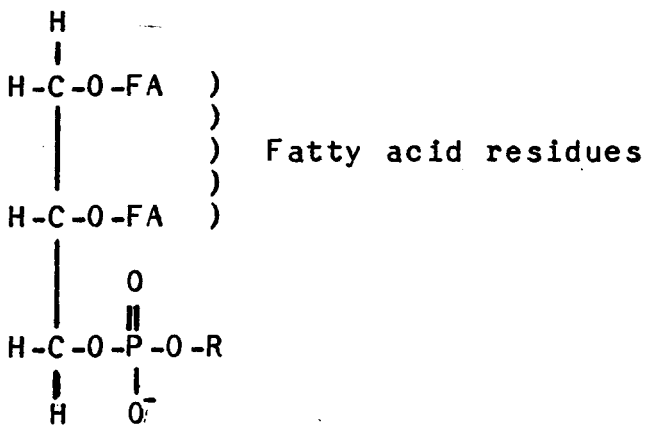
APPENDIX II

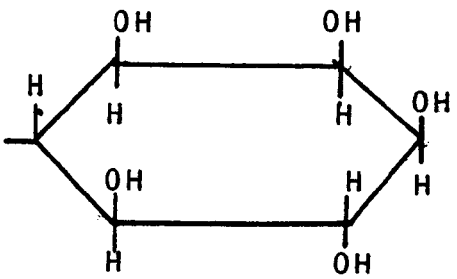
An introduction to the structure and properties of phospholipids and cholesterol.

Phospholipids are triacyl glycerols with one of the fatty acid residues substituted by a phosphoric acid residue which has an ester linkage to a polar group (usually an alcohol).

Generalised structure:-



FA are hydrophobic fatty acid residues ester-linked to the glycerol backbone. R represents a polar (alcohol) group phosphate ester-linked to glycerol and is hydrophilic in nature. Some of the major phospholipids found in natural membranes, and their corresponding R groups, are listed below:-

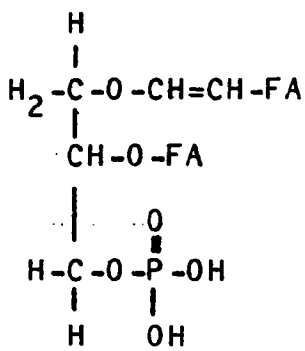
R GROUP	COMPOUND	NET CHARGE AT pH 7.0
-H	phosphatidic acid (PA)	2-
$-CH_2-CH_2-\overset{+}{N}-(CH_3)_3$	phosphatidyl choline (PC)	neutral
$-CH_2-CH_2-\overset{+}{N}H_3$	phosphatidyl ethanolamine (PE)	neutral
$-CH_2-CH(\overset{+}{N}H_3)-COO^-$	phosphatidyl serine (PS)	1-
	phosphatidyl inositol (PI)	1-
$-CH_2-CHOH-CH_2OH$	phosphatidyl glycerol (PG)	1-
$-CH_2-CHOH-CH_2-O-P(=O)(OH)-O-CH_2-CH(O-FA)-CH_2-O-FA$	cardiolipin (diphosphatidyl glycerol:DPG)	2-

The inositol lipids also include the polyphosphoinositides which are named diphosphoinositide (phosphatidyl inositol 4-phosphate) and triphosphoinositide (phosphatidyl inositol 4,5-bis-phosphate) and occur in trace amounts in most tissues. However, in nerve tissues the concentrations are higher and together these phosphoinositides account for as much

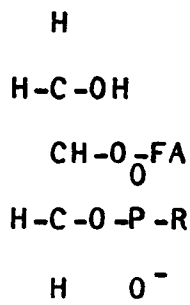
lipid phosphate as phosphatidyl inositol - about 3% of the total lipid phosphate of whole brain (Hawthorne and Kai, 1970). The functions of these lipids are mentioned in DISCUSSION.

Plasmalogens

Many phospholipids also exist in a form known as plasmalogens which contain a vinyl-ether linkage in one of the fatty acid residues, e.g., phosphatidate plasmalogen

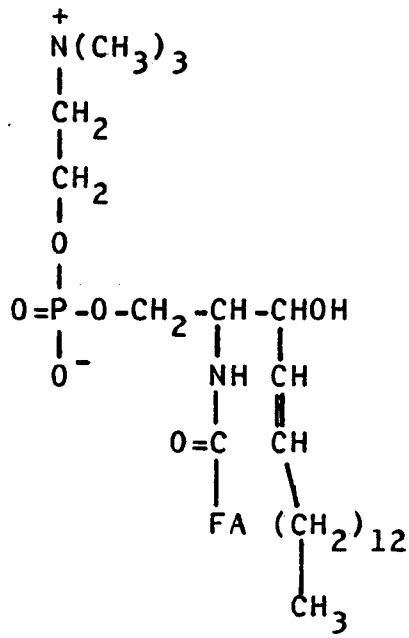


lysophospholipids also exist where one fatty acid residue is missing, e.g., lysophosphatidyl choline (LPC).



Phosphosphingolipids

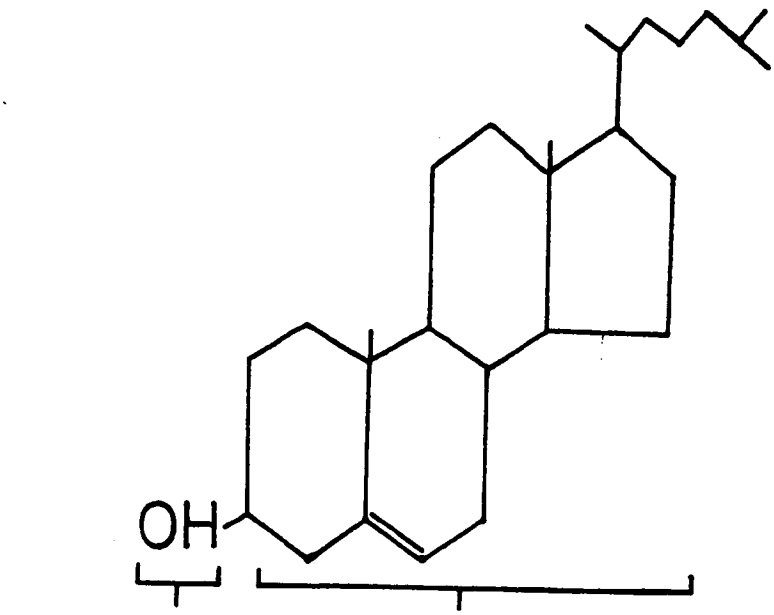
Another major phospholipid group are the phosphosphingolipids where sphingosine replaces glycerol e.g., sphingomyelin.



All of the above phospholipids are major membrane components in all types of plant or animal cells, along with a number of other lipids such as glycolipids (where a sugar moiety replaces the phospholipid polar head group and is attached directly to the glycerol hydroxyl group by a glycosidic linkage) and glycosphingolipids (where a sugar replaces the phosphate moiety of sphingomyelin).

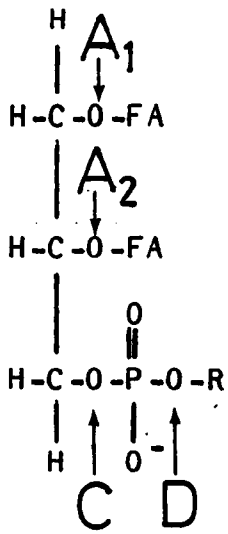
Cholesterol

Another major membrane components in higher animals is cholesterol, a neutral lipid, whose structure is outlined below:-



Phospholipid degradation

Phospholipid degradation is carried out by endogenous phospholipases whose sites of enzymic hydrolysis are shown below.



Phospholipase A_2 (V.russelli) and phospholipase C (C.welchii) are of particular interest to the present study. Phospholipase A_2 specifically removes the fatty acid at the 2-position from PC, PE and PS to yield the corresponding lyso compounds (LPC, LPE and LPS), although specificity

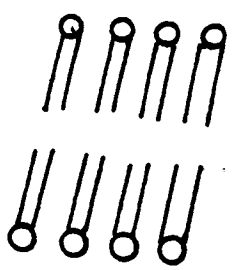
does vary from one commercial enzyme source to another (Moore and Williams, 1964; Marinetti, 1964).

Phospholipase C can attack all the major types of phospholipids with the production of a 1,2-diglyceride and a phosphatide (de Gier, de Hass and van Deenan, 1961) and is said to require Ca^{2+} for activity.

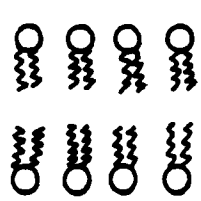
Physical Properties of Phospholipids.

Phospholipids exhibit interesting behaviour in vitro, in the presence of water. They can exist in various hydrated phases which are dependent on both the water concentration (lyotropic mesomorphism) and temperature (thermotropic mesomorphism). There are four main types of lyotropic mesophases, three of which exist when water is in excess of phospholipid.

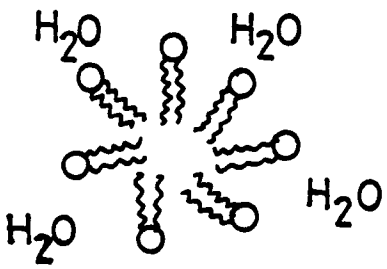
(i) Lamellar gel



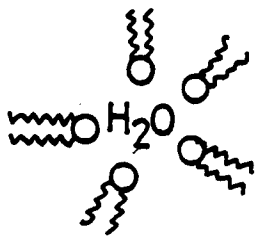
(ii) Lamellar fluid (liquid crystalline)



(iii) Hexagonal I; occurs when H_2O >> phospholipid and results in micelle formation.



(iv) Hexagonal II ; occurs when phospholipid > water.



Phospholipids have also been used as model membranes either in the form of liposomes (spherically concentric closed lamellae of 5 - 20μm diameter) or small vesicles (usually unilamellar and 0.2 - 0.5nm diameter), (Tyrrel, Heath, Colley and Ryman, 1976). However, these are essentially physical studies and it is still not yet clear exactly how they relate to <u>in vivo</u> membrane and/or lipoprotein phenomena.

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